THE JOURNAL

OF

BIOLOGICAL CHEMISTRY

VOLUME 163
BALTIMORE
1946

CONTENTS OF VOLUME 163

No. 1, April, 1946

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Letters to the Editors

THE ADSORPTION OF STRONTIUM AT FORTY DEGREES BY ENAMEL, DENTIN, BONE, AND HYDROXYAPATITE AS SHOWN BY THE RADIOACTIVE ISOTOPE

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Considerable interest in the proposed use of the radioactive isotope of strontium has arisen since Pecher (1) reported that following the administration of 8 millicuries of radiostrontium to a patient with osteoblastic metastases of prostate carcinoma, "the general condition . . . definitely improved." Pecher had used radiostrontium clinically after his animal experimentation (1–3) had shown a "selective concentration" of radiostrontium in the bones. He pointed out that the energetic character of the Sr^{89} β-rays, the favorable yields of Sr^{89} from the cyclotron, and the relatively low toxicity of strontium make Sr^{89} a possible therapeutic agent for bone tumors.

Strontium occurs in minute traces in bones and teeth; the fresh tissues contain 0.01 to 0.1 γ per gm. (4, 5). An argument that began in 1870 (6) over whether strontium can replace the calcium in bone and tooth mineral was settled in 1923 (7) when the bone ash of strontium-fed rats was found to have as little as 32 per cent calcium (normal = 37 per cent) with a concomitant increment of as much as 4 or 5 per cent of strontium. Strontium produces a peculiar fault in calcification which, as "strontium rickets" in bone and "proliferation of dentinoid" in teeth, has received careful study (8–12). However, serious toxic effects are not expected to follow low doses of soluble strontium salts (1).

Pecher (1) has shown by autoradiographs and by counting procedures that radiostrontium (like radiocalcium) is found in the soft tissues in very low percentages of the dose given either by mouth or parenterally. In contrast, the skeleton takes up large percentages; for example, in mice, 24 hours after receiving radiostrontium, 33 per cent of the dose was held in the hard tissues. Calcium is retained to an even greater degree; in an experiment parallel to the one just cited, 58 per cent of a radiocalcium dose was present in the skeleton. The distribution of strontium in the body is much like that of calcium, but both of these mineral elements differ sharply from phosphorus (13) in this respect. The latter is taken up in much higher proportions by soft tissues and much less in the skeleton; thus, only 5 per cent of a dose of radiophosphorus is found in the skeleton 24 hours

after injection. The uptake of radiophosphate by powdered bone has been described by the Freundlich adsorption isotherm (14); the data given below in detail indicate that the uptake of strontium can also be described in this fashion (Fig. 1).

The adsorption of strontium provides an adequate explanation for the mechanism by which strontium is taken up and held in the bones and teeth.
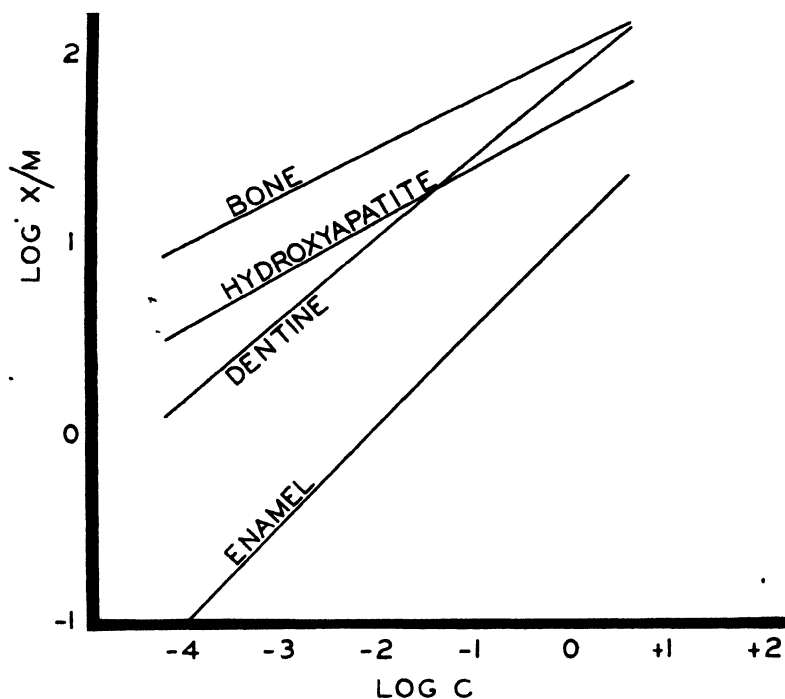
FIG. 1. Adsorption isotherms of strontium on various calcified tissues and on hydroxyapatite at 40° The curves for bone and for hydroxyapatite are nearly parallel and differ in slope from the nearly parallel curves for dentin and for enamel.

To test this hypothesis, two calculations based on the data of Pecher may be cited.[1] First, if the assumption is made that strontium in the body is handled as if it were calcium, i.e. that the calcium concentration of the blood would act as the controlling factor for strontium as well as calcium exchange in the bone mineral, the predicted adsorption from the isotherm line (Fig. 1) is 24 mg. per gm. of bone. From the percentages of administered radiostrontium actually found in the skeleton, if each radiostrontium atom represented the adsorption of strontium plus calcium in the same proportions that characterized these elements in the blood stream, it would

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[1] The bones of Pecher's mice adsorbed about 0.2 mg. of Sr per gm. of bone.

appear that the bone had taken up about 4 mg. per gm. of bone. The failure of the calculated bone value to equal the predicted adsorption value is evidence that, although strontium and calcium are distributed in similar fashions in the body, the processes of distribution are not identical and the two metallic ions are clearly differentiated by the body. Second, additional calculations may be based on the assumption that strontium is handled by the blood and bone in equilibria that are controlled by the blood strontium level. On this basis, if Pecher's mice had blood strontium levels of the order of 0.05 γ per ml.,[2] this level should define the equilibrium which has resulted in the fixation by the bone of 0.2 mg. of Sr per gm. However, from the isotherm (Fig. 1), powdered bone takes up about 3 mg. of Sr per gm. at this concentration of Sr in solution. The amount of Sr adsorbed *in vivo* is much less than would be predicted. However, these calculations contain many assumptions designed to get around the serious

TABLE I

Data on Uptake of Strontium by Various Calcified Tissues

Equilibrium, concentration of strontium (approximate)	Average strontium picked up per gm.			
	Apatite	Bone	Dentin	Enamel
M	mg	mg.	mg.	mg.
2	60	123	48	20
2×10^{-1}	34	78	32	5.3
2×10^{-2}	19	51	21	0.80
2×10^{-3}	9.8	25	11	0.53
2×10^{-4}	5.1	12	33	0.35

lack of specific data on blood strontium counts. The blood concentration has been assumed to be the same as those found in representative soft tissues; this may not be the case. In any event, the predicted values (from Fig. 1) are large enough to account for the Sr found in the bone so that a surface-limited exchange can be taken as a sufficient (although not a necessary) explanation.

The amounts of strontium which are taken up from solutions of $SrCl_2$ at 40° by powdered bone, dentin, enamel, and hydroxyapatite are given in Table I. Although a 50 mg. bone sample takes up about 6 mg. of Sr from a 2 M $SrCl_2$ solution, this represents only 0.1 per cent of the Sr in the solution. In the succeeding dilutions, increasingly larger proportions of the Sr are removed from the solutions; specifically, 0.9, 5, 24, and 36 per cent of the Sr are taken up from the dilutions, respectively, given in Table I.

It is interesting to note that for strontium, as has been repeatedly ob-

[2] Pecher did not measure the blood radiostrontium levels. This value is an approximation of the soft tissue levels, *e.g.* in the liver.

served for other ions, under the conditions of the experiment bone takes up more than dentin which, in turn, has higher values than enamel. The comparative values setting enamel at unity are given in Table II. There is a larger difference between the relative values for strontium adsorption by these tissues than for any ion reported thus far. The cause of this order of adsorption has been supposed to lie in the differences in average particle

TABLE II

Adsorptive Power of Calcified Tissues Compared to Enamel As Unity*

Ions adsorbed		Bone	Dentin
	°C.		
PO₄	40	2.4	1.3
"	200	6.3	5.2
F	40	2.3	1.9
Na	40	1.8	1.5
Sr	40	8.8	6.6

* The k values are used to compare the adsorbing power of bone, dentin, and enamel. For each ion, the ratios were obtained by dividing by the k value for enamel (1.0).

TABLE III

Adsorption of Various Ions by Calcified Tissues Compared to Strontium As Unity*

Ions adsorbed		Bone	Dentin	Enamel
	°C.			
PO₄	40	2.1	1.5	7.6
F	40	0.5	0.5	1.8
PO₄	200	0.1	0.1	0.1
Na	40	0.1	0.1	0.3
Sr	40	1.0	1.0	1.0

* The k values of the Freundlich equation are compared for the adsorption of various ions on the calcified tissues. The k values for each tissue have been converted by dividing by the k for strontium.

size; however, there is some reason to suspect that differences in density are more important perhaps because the density controls the diffusion rates for each tissue.

Comparing the k values for the ions (Table III) indicates that the adsorptive power of strontium is to be somewhat less than that of phosphate (at 40°), but markedly greater than those of fluoride, phosphate at 200°, and sodium, respectively. This order seems reasonable, since strontium would be expected to exchange easily with calcium, and the four solids studied are all calcium phosphates.

Methods

The method has been previously described in detail (14). Briefly, 50 mg. samples of powdered bone glycol ash (15), dentin and enamel (16), and hydroxyapatite Sample TR3 (17) were stirred for 45 minutes at 40° with 25 ml. of $SrCl_2$ solutions ranging in various tests from 2 to 0.0002 M. The 45 minute period of exposure to the solutions was chosen after studying the data from experiments in which exposure times of 15 to 90 minutes were employed. The solutions were decanted after centrifugation and the solids washed repeatedly for 10 minutes with distilled water at 40°. Finally the solids were dissolved in dilute hydrochloric acid and the solutions counted on our Geiger-Müller scale-of-four counters (18).

Preparation of Radiostrontium Solutions—The radioactive strontium was prepared by the bombardment of SrF_2 on a copper plate by deuterons. The nuclear reaction is

$$_{38}Sr^{88} + {}_1H^2 \longrightarrow {}_{38}Sr^{89} + {}_1H^1$$
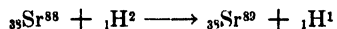
We are greatly indebted to Dr. Robley D. Evans of the Massachusetts Institute of Technology for furnishing the bombarded sample. Sr^{89} has a half life of 55 days and emits penetrating β-rays of about the same energy (maximum, 1.5 m.e.v.) as those from radiophosphorus. These properties give a great usefulness to Sr^{89} as a biological tracer substance.

During the bombardment, several isotopes of yttrium are produced; one of these (Y^{88}) has a half life of 100 days and emits penetrating γ-rays on disintegration.

The sample (copper, strontium fluoride, radioyttrium) was dissolved by repeatedly fuming off in concentrated HCl solution. The sample in a few ml. of water was added to a solution containing 20 mg. of cupric chloride, 20 mg. of yttrium nitrate, and 5 ml. of 6 N HCl, and the whole volume adjusted to 100 ml. with water. The solution was saturated with H_2S; the resulting precipitate of the "copper fraction" had 6800 c.p.m.,[3] whereas the "Sr-Y fraction" had about 800,000 c.p.m.

The filtrate was freed of H_2S by boiling; the volume was evaporated to 15 mg., the precipitate filtered out, washed, and the filtrate again evaporated to a final volume of 15 ml. An oxine solution was prepared by dissolving 0.17 gm. of 8-hydroxyquinoline in 12.5 ml. of acetone and diluting to 50 ml. with water. 7.5 ml. of this solution were added to the 15 ml. solution containing the Sr and Y and 1:1 NH_4OH solution was added until the odor of NH_3 was apparent. The solution turned dark green and a precipitate formed (yttrium hydroxyquinolate) which was filtered off. The "yttrium fraction" contained 241,000 c.p.m. and the "Sr solution fraction" about 113,000 c.p.m. per ml.

[3] Counts per minute on our scale-of-four counter.

The filtrate was evaporated to 15 ml. and 5 mg. of yttrium nitrate were added. The separation procedure was repeated, substituting 1:10 NH₄OH solution for the more concentrated one used before. The "yttrium fraction" contained 33,400 c.p.m., the "Sr fraction" about 600,000 c.p.m. Upon a third separation, the "yttrium fraction" contained 3410 c.p.m., the "Sr fraction" 578,000 c.p.m. Further repetitions of the separation were considered unnecessary. The Sr was precipitated as the carbonate and the working solution of radiostrontium was prepared by using an equivalent amount of HCl.

SUMMARY

1. Strontium is adsorbed from strontium chloride solutions by various calcified tissues and synthetic hydroxyapatite.

2. The adsorption of strontium is according to the following order: bone > dentin > enamel.

3. The adsorption of strontium by powdered bone *in vitro* is of sufficient magnitude to account for the strontium taken up by bone *in vivo*.

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ENZYMIC NATURE OF THE CAROTENE-DESTROYING SYSTEM OF ALFALFA*

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Rapid destruction of carotene occurs in alfalfa during the field curing process. The loss under good curing conditions may be 45 to 80 per cent of the carotene originally present (1–4). Hauge and Aitkenhead (5) and Hauge (6) presented evidence which indicates the presence of a carotene-destroying enzyme system in alfalfa. The belief that the destruction of carotene during field curing may be largely enzymic in character is supported by later investigations (7–10). This view has been based chiefly on the observation that autoclaving or blanching of alfalfa prevents the destruction.

The present investigation was conducted to determine the time-temperature relationships involved in the inactivation of the carotene-destroying system by heat, and to obtain additional data concerning the enzymic nature of the system.

EXPERIMENTAL

To insure a series of samples that would be uniform with respect to maturity and conditions of growth, fresh field-grown alfalfa was brought to the laboratory, the leaves were removed, thoroughly mixed, and 5 gm. portions of the leaf tissue were placed in 1 ounce bottles. The bottles were stoppered and stored at $-15°$ until used. Dry matter was determined at the time of storage.

Inactivation of System by Heat Treatment of Plant Tissue—5 gm. portions of frozen tissue were spread on a paper and defrosted at room temperature for 7 minutes. Each defrosted sample was placed in a 1 inch cube made of wire screen. This was dipped in water at a definite temperature for a definite period of time, removed, and cooled by plunging into cold water. Each sample was transferred to a bottle, stoppered, and incubated at 37° for 24 hours. Carotene was determined by a modification of the method of Moore and Ely (9). Measurements were made with a Beckman spectrophotometer at 4360 A.

The results of this experiment are presented in Figs. 1 and 2, from which it is seen that heat treatments prior to incubation greatly affect the preser-

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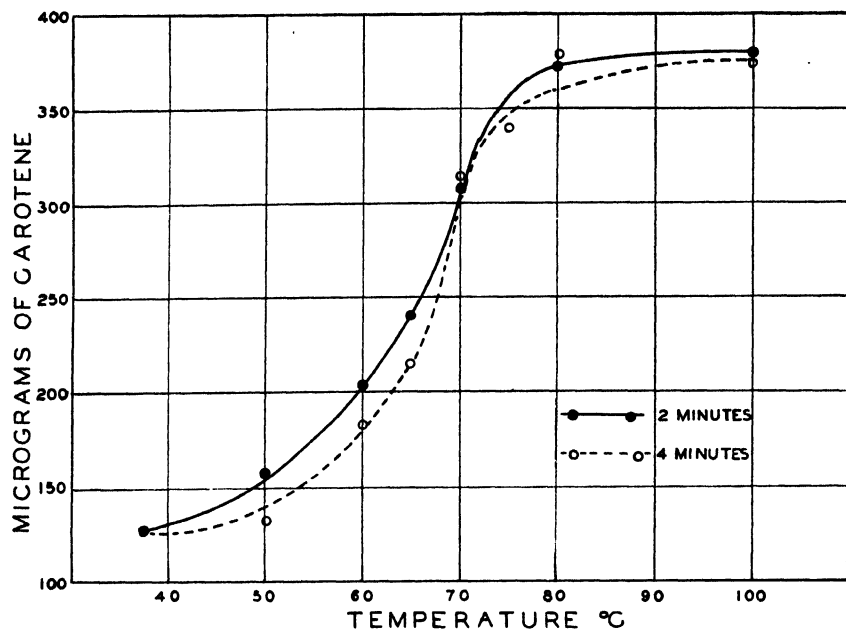
FIG. 1. The relation of heat treatment to enzyme inactivation as measured by the preservation of carotene in alfalfa leaves (initial value, 413 γ per gm. of dry matter).
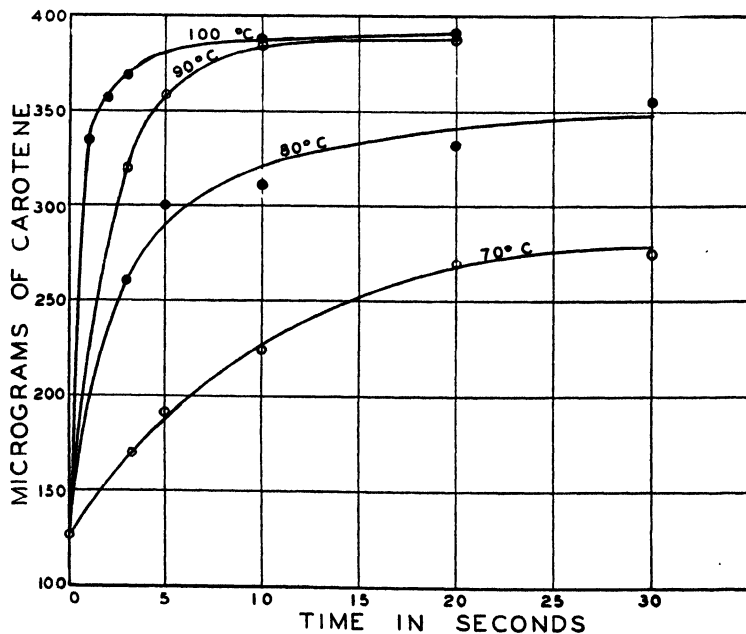
FIG. 2. Rate of inactivation of the enzymes in alfalfa leaves at different temperatures (initial value, 413 γ per gm. of dry matter).

vation of carotene. As seen in Fig. 1, the maximum inactivation of the carotene-destroying system at a given temperature was obtained in 2 minutes, since heating the tissue for 4 minutes did not result in greater preservation of carotene. The amount of inactivation increased rapidly between 65–80°.

From Fig. 2 it is apparent that inactivation is a function of both temperature and time. The carotene content of the leaves before heat treatment was 413 γ per gm. of dry matter. 89 per cent of the carotene was retained after the leaves were heated at 100° for 3 seconds and subsequently incubated, while 93 per cent was retained after treatment at 90–100° for 10 seconds. Only 55 per cent was retained after heat treatment at 70° for 10 seconds, followed by incubation.

Inactivation of System by Heat Treatment of Plant Extracts—Waugh *et al.* (9) showed that aqueous extracts of alfalfa destroyed carotene when used according to the method of Reiser and Fraps (11) for measuring the lipoxidase activity of legume seeds. The effect of heat on the activity of an alfalfa extract was studied by this technique.

An aqueous extract was prepared with a Waring blendor by dispersing 5 gm. of whole alfalfa tissue grown in the greenhouse in 200 ml. of water. Portions of the extract were heated in a water bath for 5 minutes at various temperatures. The carotene-destroying activity of each heated extract was measured by an adaptation of the method of Reiser and Fraps.

Into a 25 x 250 mm. tube were pipetted 2 ml. of the alfalfa extract, 2 ml. of phosphate buffer of pH 6.5, and 2 ml. of water. The contents of the tube were mixed and 1 ml. of a carotene solution was added. The carotene solution contained 90 γ of carotene and 0.6 mg. of Wesson oil per ml. of acetone. The tube was placed in an incubator at 37° for 1 hour. 50 ml. of heptane (petroleum ether, b.p. 90–100°) were pipetted into the tube. About 15 ml. of 95 per cent ethanol were added to facilitate extraction of the carotene from the aqueous phase by the heptane. The tube was stoppered, shaken vigorously, and the contents were transferred to a separatory funnel. Water was added to facilitate the separation of the heptane extract, after which the aqueous phase was drawn off. The heptane solution was shaken with 10 to 15 ml. of a 25 per cent solution of KOH in methanol to remove chlorophyll. The heptane was washed with water until free from alkali and was dried with anhydrous Na_2SO_4. Carotene was measured with a Beckman spectrophotometer, as previously described. A blank determination was made by substituting an equal volume of water for the alfalfa extract. Carotene-destroying activity was expressed as per cent destruction of the carotene.

The results of this experiment, presented in Fig. 3, show that aqueous extracts of alfalfa contain a carotene-destroying system which is inactivated by heat. Maximum inactivation was obtained at about 70°, somewhat

lower than the temperature of maximum inactivation of the system in the intact leaf.

Since inactivation by heat is one of the characteristics of enzyme systems, the heat lability of the carotene-destroying system strongly suggests the enzymic nature of this system.

Destruction of Carotene in Blanched Alfalfa Leaves by Added Extracts—If the material which is water-soluble and which shows activity by the Reiser-Fraps method is the same material that causes destruction of carotene in
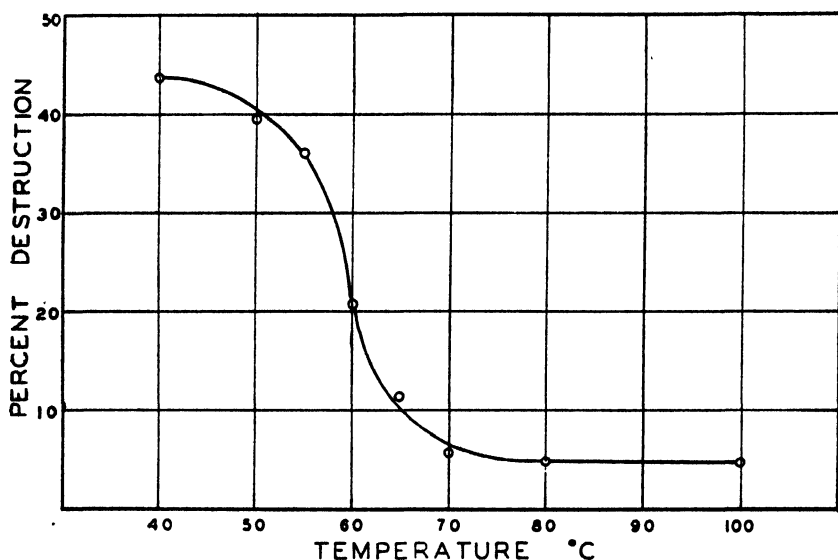
FIG. 3. Effect of heat on the carotene-destroying activity of an extract of alfalfa leaves.

alfalfa, it should cause destruction of carotene when added to alfalfa leaves in which the carotene-destroying system has been inactivated by heat.

Alfalfa juice was obtained by defrosting frozen alfalfa leaves and expelling the juice in a hydraulic press. 30 ml. of the juice were blended with 5 gm. of blanched alfalfa leaves in a small Waring blendor cup. The mixture was incubated for 8 hours at 37°. A control was prepared by blending 30 ml. of water with blanched leaves and incubating for 8 hours at 37°. Carotene was determined as previously indicated.

Since a lipoxidase has been reported by Sumner (12) to be present in alfalfa, and since soy bean lipoxidase is known to bleach carotene solutions if unsaturated fat is present (13), an experiment was conducted to determine the effect of soy bean lipoxidase upon blanched alfalfa leaves. 0.1 gm. of defatted soy bean meal was ground with 30 ml. of H_2O in a mortar. The

resulting suspension was added to 5 gm. of blanched leaves in a small Waring blendor cup and treated as described above.

The results of these experiments, as presented in Table I, show that both alfalfa juice and soy bean lipoxidase are capable of destroying carotene in blanched leaves. It can therefore be concluded that the carotene-destroying system of the leaf is soluble in water and that its solution can destroy carotene in comminuted plant tissue as well as in carotene solutions.

Agents Affecting Activity of Aqueous Extracts—Although the preceding experiment indicates that destruction of carotene in alfalfa may be due to a lipoxidase, it is possible that other substances may be present in the extract which are responsible for the destruction. A series of experiments was conducted to elucidate the enzymic nature of the system further.

TABLE I

Effect of Adding Alfalfa Juice and Soy Bean Lipoxidase to Blanched Alfalfa Leaves

Treatment	Carotene	
		Loss
	γ *per gm. dry weight*	*per cent*
Initial (no incubation).................................	512	
Blanched leaves, H$_2$O.................................	482	5.9
" " alfalfa juice...........................	289	43.6
" " soy bean lipoxidase...................	317	38.1

An extract was prepared by dispersing 5 gm. of defrosted alfalfa leaves in 200 ml. of water with a Waring blendor. The extract was filtered. 3 ml. aliquots were placed in 25 × 250 mm. tubes and the weight or volume of the various reagents as shown in Table II was added. The tubes were allowed to stand at room temperature for 1 hour, after which the carotene-destroying activity was measured both in the absence and in the presence of added oil by the adaptation of the method of Reiser and Fraps. The carotene solutions employed were: (1) 90 γ of carotene per ml. of acetone and (2) 90 γ of carotene and 0.6 mg. of Wesson oil per ml. of acetone.

In the final experiment of this series, 50 ml. of the extract were half saturated with (NH$_4$)$_2$SO$_4$ and centrifuged. Carotene-destroying activity was determined on 3 ml. of the supernatant liquid. The precipitate was dispersed in 50 ml. of the phosphate buffer (pH 6.5) and the carotene-destroying activity was determined on a 3 ml. aliquot. The results of these experiments are presented in Table II.

The protein coagulants, heat and ethanol, and the protein precipitants,

$CuSO_4$ and $Pb(OAc)_2$, caused partial or complete loss of carotene-destroying activity. Likewise formaldehyde and pancreatin, which modify the structure of proteins, reduced the activity of the system. Inactivation by NaCN and thiourea, which is characteristic of many oxidizing enzymes, is in agreement with the observation of Silker *et al.* (10) that the addition of these substances to fresh ground alfalfa tends to preserve the carotene

TABLE II

Effect of Various Treatments on Carotene-Destroying Activity of Extracts of Alfalfa

Treatment of extract		Carotene destroyed		Original activity	
Material added		No oil added	Oil added	No oil added	Oil added
	mg per 3 ml	*per cent*	*per cent*	*per cent*	*per cent*
None		36 8	53 6	100	100
Na_2SO_4	10	38 8	50 5	105 3	94 2
"	50	29 0	47.6	78 8	88 8
NaCl	10	38 3	48 9	104 0	91 2
"	50	26 3	41 4	71 4	76 9
$(NH_4)_2SO_4$	50	27 0	41 5	73 3	76 7
NaF	10	28 5	42 5	77 5	79 3
NaCN	10	2 8	0 0	7 5	0.0
Thiourea	10	11 0	17 9	29 9	33 4
$CuSO_4 \cdot 5H_2O$	10		3 1		5 8
$Pb(OAc)_2$	10	12 9	21 8	35 0	40 7
"	50		2 5		4 7
Pancreatin	50	4 0	4 2	10 9	7 8
Ethanol, 95%	(1 ml)	11 2	20 1	30 4	37 5
" 95%	(2 ")		5 9		1.1
Formaldehyde, 5%	(1 ")	15 6	19 6	42 4	36 6
$(NH_4)_2SO_4$, ppt	Half saturation		42 1		78 3
" filtrate			10 1		18 8

during dehydration. NaF, which is a strong inhibitor for hydrolytic enzymes, had relatively little effect on the system.

In contrast to $CuSO_4$ and $Pb(OAc)_2$, Na_2SO_4 and NaCl had no inhibitory action at the lower concentration studied. At the higher concentration these salts, along with $(NH_4)_2SO_4$, caused a slight reduction of activity.

The active principle can be salted-out by half saturating the extract with $(NH_4)_2SO_4$. The extract after salting-out had little activity.

The results of these experiments, together with the heat inactivation studies already discussed, clearly indicate the enzymic nature of the carotene-destroying system. A lipoxidase is shown to be present in the extract by the fact that greater destruction of carotene occurred when oil

was added than, when oil was not added. The destruction of carotene in the absence of added oil may also be due to lipoxidase activity if it is assumed that the small amount of oil which might have been dispersed in the extract from the leaves was sufficient for the reaction. If not, another enzyme is indicated. However, the effect of a given treatment in the presence and in the absence of added oil was similar when compared as percentage of the original activity. This suggests that the destruction of carotene is due to one enzyme.

DISCUSSION

The data on the relation of time and temperature to carotene preservation may be of importance to the engineer in the development of equipment for the production of alfalfa hay and leaf meal of high carotene content. To be of practical value, the heat treatment must be of short duration. This period of treatment may be less than 10 seconds at 90–100° if the heat transfer is efficient. However, preliminary experiments in this laboratory showed that inactivation could not be obtained by subjecting the plant tissue for short periods of time to a stream of air heated to 200°. The evaporation of water prevented the leaves from reaching the temperature necessary for inactivation of the enzyme. Therefore, in the design of equipment, consideration should be given to methods for the effective transfer of heat to the tissue.

SUMMARY

Evidence is presented for the enzymic nature of the carotene-destroying system of alfalfa. The enzyme involved appears to be a lipoxidase.

The enzyme was partially or completely inactivated by heat, ethanol, $CuSO_4$, $Pb(OAc)_2$, formaldehyde, pancreatin, NaCN, thiourea, and NaF, but not by Na_2SO_4 and NaCl. The enzyme was salted-out of solution by half saturation with $(NH_4)_2SO_4$.

The inactivation of the enzyme by heat was a function of both temperature and time. Maximum inactivation occurred in the plant tissue above 80° and in plant extracts above 70°. Virtually complete inactivation of the enzyme in the plant tissue was obtained at 90–100° in less than 10 seconds.

The practical application of this information is indicated.

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MANGANESE AND *l*-LEUCINE-AMINOEXOPEPTIDASE

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(Received for publication, December 20, 1945)

In recent years, it has become evident that there are at least three distinct groups of proteolytic enzymes, as judged by the conditions required for the manifestation of their activity (Bergmann (1) p. 49). One group including trypsin, pepsin, and carboxypeptidase as well as others is not known to possess any special active grouping other than amino acids. On the other hand, certain of the enzymes of both plant and animal tissues require activation by such substances as HCN, ascorbic acid, cysteine, and glutathione; this group is exemplified by papain, ficin, and some of the cathepsins. In addition to these well known enzymes, there are additional proteases whose activity is strongly enhanced by the presence of metal ions, such as Mn^{++}, Mg^{++}, Zn^{++}, and Co^{++}; this group appears to include many but not all of the peptidases present in animal and plant tissues (Johnson and Berger (1) p. 69).

The enzymes of this last group are present in active form in the tissues, as is shown by the fact that the activity of crude extracts is not increased by the addition of metal ions. However, the metal appears to be loosely bound and can be readily removed by dialysis. Much of the information regarding these peptidases has been obtained on dialyzed crude extracts but a few studies have been made on highly purified enzyme preparations. Johnson (2) has studied a peptidase from yeast which is activated by zinc or cobalt and requires the presence of halide ions. Highly purified *l*-leucine-aminoexopeptidase from intestinal mucosa is activated by manganese and by magnesium (3, 4). Both of these enzymes hydrolyze *l*-leucylglycine and *l*-leucylglycylglycine very rapidly. Some evidence has already been presented to show that the manganese activation of intestinal *l*-leucine-aminoexopeptidase is a true combination between the inactive protein and the metal ion. This is indicated by the fact that the combination of

protein with the activator metal is a time reaction. Herein, it will be shown that the activation of the enzyme is consistent with mass law considerations, and that active enzyme preparations dissociate on dilution in the absence of excess manganese. We have recently had the opportunity to study the electrophoretic behavior of our enzyme preparations and these experiments also bear on this problem.

<center>EXPERIMENTAL</center>

Enzyme activity was determined as previously described (4). Activity was determined from the first order velocity constants K where $Kt = \log a/(a - x)$ at enzyme concentration E expressed as mg. of protein N per cc. of test solution. The proteolytic coefficient $C = K/E$. Substrate hydrolyses were carried out in 2.5 cc. volumetric flasks at 40°, and were measured on 0.2 cc. samples by the titration method of Grassmann and Heyde (5). *l*-Leucinamide was used at a concentration of 0.05 M and *dl*-leucylglycine at 0.1 M. Solutions were buffered by the addition of 0.5 to 1.0 ml. of 0.1 M veronal buffer to the test solution. Incubations of Mn^{++} with enzyme in the absence of substrate were carried out in 5 or 10 cc. volumetric flasks containing 3 to 6 cc. of veronal buffer.

The purified enzyme preparations used in these studies have already been described (4). These preparations show less than 2 per cent of their maximal activity in the absence of added manganese. Reactivation of the enzyme is a time reaction which requires many hours to attain first order kinetics with *l*-leucylglycine or *l*-leucylglycylglycine as the substrate. Concurrently, some loss of activity occurs. However, with *l*-leucinamide the data more closely approach first order kinetics after incubation of Mn^{++} with the protein for 3 hours at 40° and pH 8.0. This substrate is more suitable for kinetic studies even though the enzyme hydrolyzes the amide at about half the rate that it hydrolyzes the di- or tripeptides. All of the evidence available at present indicates that *l*-leucinamide and *l*-leucylglycine are hydrolyzed by the same enzyme, and that activation or destruction of activity towards one substrate is accompanied by a parallel change towards the other substrate.

Effect of Manganese Concentration—Since the enzyme consists of a labile, protein-metal compound, a study was made of the equilibrium constant of the reaction

$$\text{Protein} + Mn^{++} \rightleftharpoons (Mn^{++}\text{-protein}) \qquad (1)$$

by varying the Mn^{++} concentration at constant protein concentration. The data for such an experiment are presented in Table I. The results show the characteristic data for an equilibrium of the kind postulated by Bergmann ((1) p. 49), and can be described by the general mass law equation

$$K_a = X/(A - X) \; [Mn] \qquad (2)$$

TABLE I

Effect of Manganese Concentration on l-Leucine-aminoexopeptidase

A constant amount of enzyme (protein N, 72.0 γ per cc.) was incubated with various concentrations of $MnSO_4$ for 3 hours at 40° and pH 8.0. A sample of each mixture was then added to the test solution of l-leucinamide. The enzyme concentration was 11.5 γ of protein N per cc. in the reaction mixture. The concentrations of Mn^{++} given below are for the reaction mixture; the incubation mixture contained 6.25 times as much. The two separate sets of data were obtained on successive days.

$[Mn^{++}] \times 10^5$	Time	Hydrolysis	C_{LA}*	C_{LA} average	C_{LA} calculated
moles per l.	min.	per cent			
4.0	75	73	0.66		
	80	83	0.74		
	105	87	0.73	0.71	0.65
8.0	60	74	0.85'		
	75	84	0.92		
	90	85	0.80	0.86	0.85
16.0	75	85	0.96		
	90	90	0.97	0.97	1.01
80.0	30	59	1.12		
	45	77	1.23		
	60	85	1.19	1.18	1.21
800.0	30	60	1.15		
	45	79	1.31		
	60	88	1.33	1.26	1.27
0.32	60	19	0.13		
	90	30	0.15		
	120	42	0.17	0.15	0.10
0.32	75	26	0.15		
	90	31	0.16		
	120	42	0.17	0.16	
0.80	30	14	0.19		
	60	27	0.20		
	90	41	0.22		
	120	55	0.25	0.22	0.23
0.80	60	28	0.21		
	90	41	0.22		
	120	56	0.26	0.23	
1.60	30	23	0.33		
	60	44	0.37		
	90	65	0.44		
	105	74	0.49	0.41	0.38
32.0	15	32	0.97		
	30	56	1.03		
	45	72	1.07	1.02	1.12
320.0	15	41	1.32		
	30	66	1.36		
	45	80	1.35	1.34	1.26

* Proteolytic coefficient for l-leucine-aminoexopeptidase.

where X is the amount of enzyme (proportional to C_{LA}) present at different Mn^{++} concentrations, A is the maximal enzyme activity (proportional to maximal C_{LA}), and K_a is the association constant. In the last column of Table I are given the calculated values of C_{LA} from Equation 2 where A is 1.27 and K_a is 25,000 molar^{-1} (K_d, the dissociation constant, is 4.0 $\times$ 10^{-5} molar where $K_d = 1/K_a$). The calculated values show that the suggested mechanism of a dissociable metal-protein compound is in agreement with the data.

While the data of Table I for the separate hydrolyses have been calculated on the basis of first order kinetics, there is some uncertainty that this mechanism applies, particularly at low Mn^{++} concentrations when the data show increasing values of first order constants during the course of the measurements. This might be interpreted as showing that activation is incomplete. A second possible explanation of the aberrant results at low Mn^{++} concentrations is that the substrate concentration is high enough to saturate the small number of active enzyme molecules, thus resulting in a zero order mechanism. The data at the lowest Mn^{++} concentrations are indeed well described by the zero order mechanism. It is possible that the conventional use of synthetic substrates at 0.05 M concentration may be too high for first order kinetics with this enzyme when low concentrations of active enzyme are employed. However, these factors, completeness of activation and relative concentrations of substrate and enzyme, do not influence the conclusions drawn regarding the activation mechanism by Mn^{++}. The concentration of active enzyme at any value of Mn^{++} can be computed by using the reciprocal of the time necessary to effect a constant quantity of substrate hydrolysis. From the relationship of active enzyme at different Mn^{++} concentrations, the data obtained are well described by Equation 2 and yield essentially the same dissociation constant; for 50 per cent hydrolysis, $K_d = 4.8 \times 10^{-5}$ and for 75 per cent hydrolysis, $K_d = 4.0 \times 10^{-5}$.

With leucylglycine as the substrate, the kinetics of the enzyme are complicated by a greater degree of zero order kinetics at low enzyme concentration and a longer activation time. It was therefore not possible to obtain satisfactory quantitative data, but the same general effect of varying Mn^{++} concentration was found. With Mg^{++} as the activating metal, complete activation of the enzyme could not be obtained even with 24 hours activation at pH 8.0 and 40°. With both Mn^{++} and Mg^{++} lower pH values decrease the rate of activation; higher pH values result in the formation of large amounts of insoluble hydroxides. Nevertheless, the data obtained with Mg^{++} showed that at least 10-fold higher concentrations of this metal were required to effect maximal activation as compared with Mn^{++}.

Effect of Enzyme Concentration on Activity—Evidence of the lability of the active enzyme can also be obtained by another method. A large amount of active enzyme was formed by incubating the protein with sufficient Mn^{++} to produce maximal activation. Different quantities of this incubation mixture were then added to the test substrate. If the active enzyme were completely stable, the proteolytic coefficients (C) should be constant, indicating a strict proportionality of activity to enzyme concen-

TABLE II

Dissociation of l-Leucine-aminoexopeptidase by Dilution

A buffered solution of $MnSO_4$ (0.02 M) and protein (36 γ of protein N per cc.) was incubated for 3 hours at 40° and pH 8.0. Aliquots of the incubation solution were then added to the test solution of *l*-leucinamide.

[Mn++] × 10⁴	Protein N	Time	Hydrolysis	C	C average
moles per l.	*γ per cc.*	*min.*	*per cent*		
4.0	0.72	90	11	0.76	
		150	19	0.83	
		210	24	0.79	
		240	27	0.79	0.79
8.0	1.44	90	24	0.93	
		150	37	0.93	
		210	47	0.92	
		240	51	0.90	0.92
16.0	2.88	30	20	1.12	
		60	31	0.94	
		120	55	1.01	
		150	64	1.03	1.03
24.0	4.32	30	29	1.15	
		45	40	1.15	
		60	51	1.20	
		75	55	1.07	
		120	78	1.27	1.17
40.0	7.20	30	42	1.09	
		60	66	1.09	
		75	77	1.18	1.12

tration, or if the amount of enzyme were high in relation to substrate concentration, the relative C values should decrease with increasing enzyme concentration (Van Slyke (1) p. 33). Actually, the data in Table II indicate that at the lower enzyme concentrations there is a deficit of enzyme activity. Since satisfactory first order velocity constants are obtained at each enzyme concentration, there is no appreciable inactivation of the enzyme during the measurements. This shows that the enzyme is stable at low protein concentrations and the lack of proportionality between protein concentration and activity must be due to some

other cause. The decrease in activity at low concentrations can be interpreted as due to a dissociation of the active enzyme into its component parts, protein and manganese. The dissociation appears to be instanta-

TABLE III

Enzyme Concentration and Activity after Prolonged Incubation

The protein (6.63 γ of protein N per cc.) was incubated with 0.01 M MnSO$_4$ at 40° and pH 7.9. Aliquots were then pipetted into the reaction flasks containing *dl*-leucylglycine (0.1 mM per cc.).

Time of incubation	Protein N	[Mn^{++}] $\times$ 10^4	Time	Hydrolysis	C_{LG}	C_{LG} average
hrs.	γ *per cc.*	*moles per l.*	*min.*	*per cent*		
0	2.65	40.0	30	18	1.09	
			60	41	1.43	
			90	70	2.19	
			150	100		
3	2.65	40.0	30	30	1.96	
			60	51	1.96	
			90	72	2.30	
17	0.265	4.0	150	4	0.43	
			270	6	0.35	
			360	7	0.35	0.38
	0.663	10.0	150	10	0.46	
			270	16	0.42	
			360	21	0.44	0.44
	1.33	20.0	150	17	0.40	
			270	27	0.39	
			360	37	0.42	0.40
	1.99	33.3	150	25	0.42	
			210	32	0.40	
			270	38	0.39	
			360	51	0.43	0.41
	2.65	40.0	30	7	0.42	
			60	14	0.41	
			90	23	0.48	
			210	47	0.50	
			270	55	0.48	0.46
	3.98	60.0	150	44	0.43	
			210	56	0.43	
			270	68	0.46	0.44

neous within the time limits of these experiments. These data for the dissociation of the enzyme can be described by Equation 2.

Enzyme activity proportional to concentration is observed with the peptidase over a wide range of enzyme concentrations, provided the ratio of Mn^{++} to protein is extremely high. Under these conditions, the tendency to dissociation is repressed. Such an experiment is presented in Table III in which *dl*-leucylglycine is used as the substrate. Incubation

of the protein and Mn^{++} was for 17 hours at 40°. This resulted in the inactivation of about 80 per cent of the activity for this substrate but the reaction between manganese and protein is complete. The data in Table III also show the characteristic rising constants when there is no incubation of protein and Mn^{++}.

For the experiments at higher enzyme concentrations (Table I), the activity would be expected to remain constant at manganese concentrations higher than 0.002 M, whereas at the lower enzyme concentrations (Table III) the activity is constant at 0.0004 M manganese. It can be predicted that the effect of manganese concentration at different protein concentrations should produce a family of curves in which the apparent dissociation constant should vary with the protein concentration. The dissociation constant calculated for the data in Table I therefore holds only for the particular protein concentration.

It should be remarked that the situation which has been found to apply to this enzyme is not unique. Dissociation phenomena leading to apparent lack of proportionality between the amount of enzyme and the measured activity will be found for all enzymes which contain a dissociable prosthetic group. Such instances may be observed in the data recorded by Irving, Fruton, and Bergmann (6) for the enzymes papain and ficin. These enzymes showed strict proportionality of velocity constants to enzyme concentration when cysteine was used as the activator. However, when HCN was used as the activator with either of these enzymes, or when ficin was studied without added activator, the velocity constants increased more rapidly than did the enzyme concentration. These data indicate that at the lower enzyme concentrations dissociation of an enzyme-activator complex occurred and the activator was actually at too low a concentration to maintain complete activation. It should be possible to measure the dissociation constants of these enzymes for their different activators, and it can be predicted that cysteine-papain and cysteine-ficin have smaller dissociation constants than their corresponding HCN enzymes. These data give an additional indication that cysteine, HCN, and other "activators" do not act only by effecting some change such as reduction in the enzyme. Data of the kind cited can best be explained by the existence of labile equilibria of the enzyme and prosthetic group. This is in complete accord with the equilibria postulated by Irving, Fruton, and Bergmann (7) for the activation of papain-β-trypsinase.

Manganese and Enzyme Stability—Van Slyke ((1) p. 33) has pointed out that part of the activating effect of manganese on arginase described by Hellerman and Stock (8) may be due to the increased stability of the enzyme in the presence of the metal ions. If such were the case for the enzyme used in these experiments, it might speak against the existence of a true compound of Mn^{++} and protein. It has already been pointed out

that the metal-free protein is, at the same temperature and pH, much more stable than is the Mn^{++}-protein compound (4). It was therefore of some interest to examine the stability of the leucine-aminoexopeptidase at different manganese concentrations (Table IV). For the short incubation (3.5 hours), the enzyme is not fully activated and it is not possible to compute precise proteolytic coefficients. Nevertheless, during the 96 hour incubation it may be computed that more than 95 per cent of the activity is lost at the two higher manganese concentrations, while at the lower concentrations there is no apparent loss of activity. The apparent complete stability under the latter circumstances is somewhat fictitious. Since only

TABLE IV

Stability of l-Leucine-aminoexopeptidase at Different Manganese Concentrations

Incubation of protein (33.1 γ per cc.) was at 40° and pH 8.0 at concentrations of $MnSO_4$ 12.5 times those given below. The samples were then tested for activity with *dl*-leucylglycine as the substrate. The enzyme concentration in the test mixtures was 2.65 γ of protein N per cc.

Time of incubation	$[Mn^{++}] = 8 \times 10^{-4}$		$[Mn^{++}] = 8 \times 10^{-5}$		$[Mn^{++}] = 8 \times 10^{-6}$		$[Mn^{++}] = 2 \times 10^{-6}$		No added Mn	
	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis	Time	Hydrolysis
hrs.	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*	*min.*	*per cent*
3.5	30	29	30	15	60	10	90	9	210	0
	60	52	60	27	90	19	180	23		
	90	73	90	41	180	42	210	26		
	210	91	210	84	210	51				
96.0	120	5	120	2	120	31	120	12	360	2
	270	8	270	4	270	49	270	28		
	360	8	360	7	360	54	360	37		
Activity remaining, %	Less than 5		Less than 5		More than 95		*Ca.* 100			

about 10 to 20 per cent of the enzyme is activated, it will be this activated enzyme which is destroyed and more active enzyme will form by combination with manganese in accordance with the equilibrium postulated in Equation 1. As long as there is a sufficient reserve of unactivated protein, there will be no apparent loss of activity, but when this reserve is exhausted, the measured amount of enzyme will decrease.

Electrophoretic Analysis—Recently, the opportunity was available for electrophoretic analysis of two of our enzyme preparations. These preparations were made in 1941. Preparation C was prepared as previously described (4) by precipitating the extract of an acetone-dried powder with ammonium sulfate (40 to 60 per cent saturation). Preparation D was

collected from the supernatant of Preparation C by raising the ammonium sulfate concentration to 80 per cent of saturation. Both Preparations C and D were thoroughly dialyzed in the cold against distilled water, the insoluble precipitates discarded, and the clear filtrates dried *in vacuo* from the frozen state and held in the desiccator. After 4 years, no loss in solubility had occurred. Electrophoresis was performed in a Tiselius apparatus equipped with the Longsworth scanning device (9). Only the descending boundaries were used for measurement. The runs were performed at 1° in veronal buffer of an ionic strength of 0.1.

The data for two runs are presented in Table V. Preparation C which had a proteolytic coefficient (C_{LG}) of 1.9 showed the presence of three com-

TABLE V

Electrophoresis of Preparations of l-Leucine-aminoexopeptidase

Electrophoresis was performed at 1° with veronal buffer of ionic strength 0.1. pH values were measured with a glass electrode at 25°. Mobilities are in sq. cm. per volt second.

Preparation	C_{LG}	Con-centra-tion	Component										pH
			u × 10⁵		u × 10⁵		u × 10⁵		u × 10⁵		u × 10⁵		
		per cent		*per cent*		*per cent*		*per cent*		*per cent*		*per cent*	
C. 40–60% saturated $(NH_4)_2SO_4$	1.9	0.8					4.3	42	5.6	17	6.8	41	8.33
D. 60–80% saturated $(NH_4)_2SO_4$	1.2	1.2	2.3	2	3.1	13	4.7	23			6.6	62	8.50

ponents. Preparation D which was of lower activity had even more boundaries. Comparison of the two preparations indicates that the component of mobility 4.3 to 4.7 is the only one which bears a relationship to the enzyme activity. It is therefore likely that the enzyme activity is associated with this component. If it is assumed that all of this component is the enzyme, it may be calculated that the homogeneous enzyme, would have a proteolytic coefficient (C_{LG}) of 4.5 calculated from the 42 per cent purity of Preparation C. It should be remarked that the highest activity reported for any proteolytic enzyme is 8.5 for carbobenzoxyglycyl-*l*-phenylalanine (10). The pure *l*-leucine-aminoexopeptidase would therefore be expected to have an activity of approximately the same order of magnitude.

Preparation C was redialyzed with the same buffer plus 0.001 M Mn^{++} and run in the electrophoresis apparatus as before. Distinct changes in mobility were found: one component changed from 4.3 to 3.8 × 10⁻⁵, and

the fastest from 6.8 to 5.3 $\times$ 10^{-5}; the intermediate component could no longer be distinguished, and appeared to be included with the other two. With the smaller difference in the mobility of the components, the separation was no longer as distinct as earlier in the short time (150 minutes) during which good observation was possible at the low protein concentration. Similar decreases in the mobilities were found when the electrophoretic behavior of Preparation D was observed in the presence of 0.001 M $MnSO_4$.

The observed drop in the mobilities suggests a suppression of carboxyl groups and is consistent with the view that the combination of Mn^{++} with the proteins is non-ionic in nature, which is also indicated by the long activation time of the enzyme. Since it is known that electrophoretic mobility may be correlated with the titration curves of various proteins (11, 12), the observed mobility changes are in agreement with observations of Main and Schmidt (13) on the combination of Mn^{++} with proteins and amino acids.

DISCUSSION

Mann and Lutwak-Mann (14) have pointed out in a recent review that an increased enzyme activity caused by the addition of a metal ion does not necessarily indicate that the metal is part of the active enzyme complex. They cite as an example early experiments which indicated that laccase was a manganese compound because of the activating effect of this ion, whereas the highly purified laccase of Keilin and Mann (15) is a copper-protein. It is felt that this is a timely warning and the evidence presented in this paper has been examined in the light of this statement. If the action of the metal ion were not due to formation of a true metal-protein enzyme, there are several possible modes of action of the metal. One way would be by combination with a specific inhibitor; a second possibility would be by stabilization of the enzyme, in the same way that colloids such as protein or gum arabic in high concentrations are known to stabilize many enzymes. These two possibilities will be examined below, and it will be demonstrated that specific evidence can be adduced to rule out both of these ideas.

As evidence for the fact that *l*-leucine-aminoexopeptidase is an easily dissociated metal compound (Mn^{++} or Mg^{++}) the following may be summarized.

1. Purification of the enzyme leads to a progressively greater activation by metal (4). If the rôle of the metal is one of combining with some inhibitor, it should be expected that the proportionate amount of inhibitor should decrease on a purification of 30- or 40-fold in terms of protein N and many times higher on a dry weight basis. Dialysis would be expected to remove a low molecular weight inhibitor; actually dialysis causes a decrease in enzyme activity.

2. The activation of the enzyme with metal is a time reaction which requires some hours for completion under ordinary experimental conditions. During the course of activation, the rate of hydrolysis shows a steady increase. Such an increase in enzyme activity cannot be explained in terms of stabilizing influence.

3. The metal ion does not produce any apparent stabilization, since the fully activated enzyme is more easily inactivated at 40° and pH 8.0 than is the unactivated enzyme. (Also see Schwimmer (16).) Moreover, the activated enzyme under equilibrium conditions is completely stable during the ordinary determination of enzyme hydrolysis of substrate, as shown by the fact that first order velocity constants may be obtained even over long periods of hydrolysis (Tables II and III). Likewise crude extracts which require no metal activation are much less stable than highly purified ones which do.

4. The activation of the enzyme with manganese follows the usual mass law equation.

5. Crude enzyme extracts (as well as purified preparations) are inhibited by the usual compounds which act by combining with metals; i.e., HCN, H_2S, etc. (17). The inhibition of crude extracts shows that the enzyme is present in the tissues in combination with a metal ion.

6. The activation of the enzyme is highly specific for Mn^{++} and Mg^{++}; no activation has been found for other metal ions such as iron, nickel, cobalt, zinc, and calcium. Some of these ions, so similar in chemical properties to Mn^{++} and Mg^{++}, might be expected to act in the same way towards an inhibiting substance. The activation by Mg^{++} shows that no oxidation-reduction system is involved.

7. The specific l-leucine-aminoexopeptidase is extremely wide-spread in nature (18). This enzyme regardless of source is strongly activated by both Mn^{++} and Mg^{++}.

8. There appear to be many distinct peptidases which require the presence of a metal ion for maximal activity, the purified yeast peptidase of Johnson (2) and the partially purified prolidase (4) from hog intestinal mucosa may be cited as examples. Prolidase is particularly interesting in this connection, since no metal ion other than Mn^{++} has been found to produce activation. Most of the peptidases which have been found to be strongly activated by metal ions have not been purified but there can be no doubt as to the wide-spread occurrence of metal-activated peptidases and their large number.[1]

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[1] In intestinal mucosa alone, many of the activities present in a dialyzed crude extract are strongly activated by metal ions. Simple purification steps show that most of these activities can be partially separated or differentially inactivated from one another. For example, the bulk of the activity towards l-alanylglycine (Mn^{++}-activated) is readily removed from l-leucine-aminoexopeptidase (4). The enzyme

9. Both Mn^{++} and Mg^{++} are known to form complexes with proteins and amino acids (19). Measurements on titration curves of amino acids and proteins show a distinct suppression of the ionization of carboxyl groups. Such a change in ionization is also shown by the changes in electrophoretic mobility which occur in the proteins present in the partially purified *l*-leucine-aminoexopeptidase.

In conclusion, the available evidence all points to the fact that *l*-leucine-aminoexopeptidase is a true metal-protein compound. While the rôle of the metal is as yet unknown, the fact that complete activation may require many hours indicates that some type of complex formation occurs rather than a simple ionic reaction.

SUMMARY

1. The effect of Mn^{++} on *l*-leucine-aminoexopeptidase has been described. With *l*-leucinamide as substrate, the combination of Mn^{++} and protein to form active enzyme may be described by a simple mass law equation.

2. Active enzyme dissociates to its component parts on dilution so that activity is not proportional to enzyme concentration unless a large excess of manganese is present.

3. It is shown that the stability of the unactivated protein is much greater than that of the active enzyme.

4. Some electrophoretic studies on the partially purified *l*-leucine-aminoexopeptidase are reported.

5. The available evidence for classifying *l*-leucine-aminoexopeptidase as a true Mn^{++}-protein compound and for the existence of a whole class of similar metal-protein peptidases is summarized.

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hydrolyzing *l*-glycylalanine (Mn^{++}-activated) is present in a crude prolidase preparation in greater degree than the enzyme which hydrolyzes the reciprocal peptide. It is therefore apparent that the two alanine-containing peptides are hydrolyzed by distinct enzymes. This is likewise true for the enzymes which hydrolyze glycylproline and prolylglycine and for the enzymes which hydrolyze glycylleucine and leucylglycine ((4) and unpublished observations of the author).

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ZINC TOXICITY IN RATS

ANTAGONISTIC EFFECTS OF COPPER AND LIVER

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The evaluation of the nutritional quality of foods and feeds requires a more definite knowledge of the physiological rôle of the various micro nutrient inorganic elements in the animal body than is now available. Problems of immediate concern include the effects of one or more of these elements on the utilization of another, for example the effect of traces of arsenic in reducing the toxicity of selenium (Moxon and Rhian (8)) and of copper in reducing the toxicity of molybdenum (Ferguson *et al.* (3)). It is the purpose of this paper to summarize studies of an anemia and subnormal growth induced in rats by high zinc diets and the antagonistic effects of copper and liver supplements.

Ferguson *et al.* reported that certain "teart" pastures in England which were toxic for cattle contained a large amount of molybdenum and that this molybdenum toxicity could be prevented by drenching the cattle with a solution of copper sulfate. Such observations as well as those to be reported in this paper point to the possibility of deficiencies occurring in grazing animals, which are relative rather than absolute. It is usually concluded that if "sick" animals respond favorably to dietary supplement, such as copper for example, the animals and the feed they consumed were copper-deficient and copper therapy is advised. It may be that such therapy only partially corrects the condition, for, as pointed out in this study and in that of Ferguson *et al.*, copper deficiency may be relative to a high content of some other factor in the feed which renders a normally adequate copper content inadequate.

The present study was stimulated by the report of Sutton and Nelson (11) who observed that excess dietary zinc produced an anemia, subnormal growth, and reproductive failure in rats. These observations were fully confirmed by the authors. Further studies showed that feeding a mixture of iron, copper, and cobalt salts largely or completely prevented the anemia and that a supplement of liver extract partially prevented the subnormal growth. These preliminary observations led to more detailed experiments which are reported below.

Methods

The basal diet used in these studies was a commercial calf meal which has proved over many years to be an adequate stock diet for rats (Maynard (7)). Zinc was thoroughly mixed into this calf meal as c.p. zinc carbonate and fed *ad libitum* to the rats. The supplements of liver extract[1] and iron, copper, and cobalt salts were mixed in a few ml. of milk and fed to the animals daily in Pyrex glass cups. When these supplements were fed, the daily amounts were 2 mg. of iron as ferric pyrophosphate, 0.2 mg. of copper as cupric sulfate, 0.2 mg. of cobalt as cobaltous chloride, and 1 gm. of liver extract. The rats were from our colony which originated from Sprague-Dawley stock. Young rats ranging from 4 to 6 weeks of age were started on each experiment. They were kept in individual wire screened cages during the studies which usually lasted from 4 to 6 weeks. Hemoglobin levels were determined periodically on blood samples secured by clipping the tails. At the end of the experiment approximately 0.5 ml. of blood was removed by heart puncture, mixed with heparin to prevent coagulation, and subjected to various determinations in duplicate. Hemoglobin was determined in a photoelectric colorimeter as oxyhemoglobin (Sheard and Sanford (9)). Red cells and leucocytes were counted in the usual manner. The red cell volume (centrifuge hematocrit) was determined with the modified centrifuge tubes described by Gruneberg (Smith (10)). Blood smears were prepared for microscopic study by staining with hematoxylin and eosin.

Results

In a series of preliminary experiments in which a level of 1 per cent of zinc in the diet was used, the following observations were made. Hemoglobin decreased to levels of severe anemia in 3 to 5 weeks. The rats eventually stopped growing and many of them died before the 6th week, particularly the youngest rats of the group. The hair was unkempt; the rats walked with a highly arched back and were very irritable when handled. If in addition to the high zinc diet a mixture of iron, copper, and cobalt salts was fed, the hemoglobin remained at or near to normal levels over the 4 to 6 week period, but body weight remained subnormal. If in addition to the 1 per cent zinc diet liver extract was fed, the hemoglobin dropped to levels comparable with those of rats fed the high zinc diet alone; however, growth was maintained near the normal level and the rats lived for longer periods of time. It thus appeared that the excess dietary zinc had at least

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[1] Liver extract powder, Eli Lilly and Company.

two effects: (1) it produced an anemia, and (2) it produced a subnormal rate of growth that was not secondary to the anemia.

Two factorially designed experiments were then conducted. One was designed primarily to study the effects of iron, copper, cobalt, and liver extract on the course of the anemia, which for convenience will be referred to as "zinc anemia." The other experiment was designed to study the effect of liver extract on the subnormal growth induced by the feeding of

TABLE I

Mean Hemoglobin Values of Group on Each Treatment

Treatment	Mean Hb value* per 100 ml. blood
	gm
None	10.64
Fe	13 02
Cu	14 04
Fe, Cu	13 84
Co	11 90
Fe, Co	11 06
Cu, "	14 24
Fe, Cu, Co	14 92
Liver	13 80
Fe, liver	13 10
Cu, "	14 46
Fe Cu, liver	15 40
Co, liver	14 70
Fe, Co, liver	14 24
Cu, " "	14 50
Fe, Cu, Co, liver	15 62

* Mean of five rats

excess zinc. Detailed studies were made of the blood picture of rats in the second experiment.

Zinc Anemia—The 1 per cent level of zinc used in the preliminary experiments was so toxic that many (up to 75 per cent) deaths resulted in 3 to 5 weeks. A pilot study was conducted testing the effect of various levels of zinc on the decrease in hemoglobin level. By feeding zinc at various levels ranging from 0.4 to 1.0 per cent of the diet it was found that a level of 0.7 per cent produced a marked anemia in 4 weeks and permitted the rats to live for relatively long periods of time. Accordingly, a level of 0.7 per cent zinc was chosen for the next experiment which involved the dietary supplements of iron, copper, cobalt, and liver extract, each at "high" and "low" levels in all possible combinations. The high level

indicates the addition of the supplement in amounts previously mentioned, and the low level indicates no addition of the supplement to the high zinc diet.   This was a 2^4 factorial experiment with five replications.   80 rats of mixed sexes, ranging in age from 30 to 40 days and in weight from 36 to 71 gm., were divided at random (Fisher and Yates (4)) into sixteen groups of five animals each.   Sixteen animals, one animal receiving each treatment, constituted a block.   The rats in each block were arranged at random in a battery of cages, giving five batteries or blocks.   The five

TABLE II

Analysis of Variance of Hemoglobin Levels of Rats Fed Various Supplements in Addition to High Zinc Diet

Source of variation	df	Mean square
Total	79	
Replications	4	8 34*
Fe	1	4 90
Cu	1	76 05*
Co	1	4 80
Liver	1	38 64*
Fe × Cu	1	0 39
" × Co	1	2 74
" × liver	1	0 00
Cu × Co	1	0 22
" × liver	1	8 58†
Co × "	1	2 38
Fe × Cu × Co	1	8 06†
" × " × liver	1	3 28
Cu × Co × "	1	7 20
Fe × " × "	1	0 92
" × Cu × Co × liver	1	3 04
Error	60	1 83

   * Significant at the 1 per cent level
   † Significant at the 5 per cent level

batteries were distributed in the air-conditioned animal room, and differences between batteries served as a measure of positional effects.   The rats were weighed weekly during the experiment, which was terminated at the end of 6 weeks.   At this time blood samples were taken by heart puncture, and the hemoglobin level determined.   The mean hemoglobin values of the different groups are summarized in Table I.   Table II presents a summary of the analysis of variance of the hemoglobin values.

   The analysis of variance showed that the rats fed the high zinc diet plus copper or liver extract had higher hemoglobin levels than those rats fed the high zinc diet without added copper or liver extract.   These differences

were highly significant.   Also, there was a significant interaction of copper and liver extract due to the fact that copper supplements produced a greater response in the hemoglobin level in the absence of liver than it did in the presence of liver extract.   There was a significant interaction of iron by copper by cobalt, which resulted in a higher hemoglobin level than would be expected if additive effects of the metals fed singly or in combinations of any two were involved.   There was a highly significant difference between replications, indicating that some factor or factors associated with
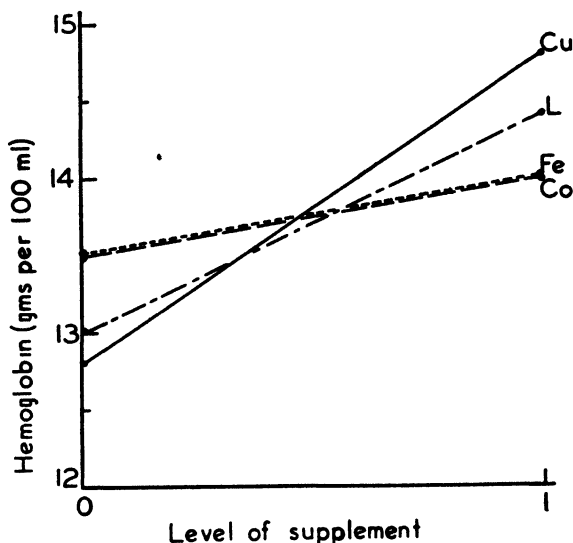
FIG. 1   The hemoglobin level of the rats fed the various supplements at 0 and 1 levels.   The 0 level indicates that the supplement was not fed and the 1 level that it was fed.   Each point is the average hemoglobin level of forty rats.   The difference between the hemoglobin levels of the rats not fed additional copper salts and those fed copper salts in addition to the high zinc diet is highly significant.   The same holds true for the difference between the rats not fed liver extract and those fed liver extract (L)

the position of the five replications introduced a difference in hemoglobin levels that was highly significant.   Since the room was air-conditioned and maintained at a temperature of 24.4°, the factor probably is not temperature variation.   All other sources of variation were non-significant.

The hemoglobin levels observed at high and low levels of iron, copper, cobalt, or liver extract are graphically presented in Fig. 1.

Growth—In the foregoing experiment the effects of the various treatments on growth were tested by an analysis of variance, and the addition of liver extract to the diet was associated with significantly better growth (odds 19:1) than was the case for those rats fed the basal diet plus 0.7 per cent

zinc. However, previous observations indicated that a much more marked effect could be obtained with liver extract when the higher levels of zinc were fed. Accordingly, a second factorial experiment was set up involving a basal diet containing 1 per cent of zinc and supplemented with liver extract and the mixture of iron, copper, and cobalt salts, each at a high and low level. The mixture of iron, copper, and cobalt salts was chosen, since it most effectively prevented a drop in hemoglobin level among the various combinations of the elements. This was a 2^2 factorial experiment with ten replications.

Forty rats, ranging in age from 26 to 29 days and in weight from 42 to 82 gm. and consisting of twenty males and twenty females, were allotted at random to the four groups treated differently, leaving five male and five female rats in each group. The rats were caged individually and distributed at random on five shelves of a cage battery, so that each shelf con-

TABLE III

Mean Initial and Final Weights of Rats Fed High Zinc Diet and Various Combinations of Liver Extract and Iron, Copper, and Cobalt

Treatment	Mean initial weight*	Mean final weight*
	gm.	*gm.*
None................	61.5	118.5
Liver.................................	62.7	155.1
Fe, Cu, Co.....	64.5	120.8
Liver, Fe, Cu, Co...........	64.3	148.7
Stock diet...	62.4	174.6

* Mean weights of ten rats (five males and five females).

tained eight rats, one on each treatment, resulting in five replications. The rats were weighed weekly during the experiment which ran for 5 weeks. At the end of this time, blood samples were taken from all rats except the group which received all supplements; that is, iron, copper, cobalt, and liver extract. Red cell counts, leucocyte counts, hemoglobin, and hematocrit determinations were made. A group of ten comparable rats which received the calf meal alone (stock diet) was also studied to provide control values for comparison.

The mean initial and final weights of the rats on each treatment are summarized in Table III. The analysis of variance of the growth data is summarized in Table IV.

It will be noted that the liver extract supplement to the high zinc diet induced a positive growth response which when compared to the growth of rats fed the high zinc diet alone gave a difference that was highly significant. Although the supplement of iron, copper, and cobalt maintained hemoglobin at levels significantly higher than in those rats fed the high

zinc diet alone (Table V), it had no effect in counteracting the subnormal growth induced by the excess dietary zinc.   A significant (99:1) sex differ-

TABLE IV

Analysis of Variance of Growth Data of Rats Fed High Zinc Diet and Various Combinations of Liver Extract and Mixture of Iron, Copper, and Cobalt

Source of variation	df	Mean square
Total	39	
Replications	4	703
Sex	1	9,456*
Replications × sex	4	1,664
Liver	1	10,401*
Fe, Cu, Co	1	42
Sex × liver	1	2,512*
Liver × Fe, Cu, Co	1	189
Error 1	26	558
" 2†	28	615
" 3‡	32	746

* Significant at the 1 per cent level
† Used to test the significance of liver and Fe, Cu, and Co
‡ Used to test the significance of replications and sex

TABLE V

Blood Pictures of Rats Fed the Zinc Diet Alone or Plus Liver Extract or Fe, Cu, and Co Supplements along with Those for Comparable Rats Fed a Stock Diet

Treatment	Mean Hb	Mean r b c	Mean hematocrit	Mean cell volume	Mean cell Hb concentration	Mean cell Hb	Leucocyte count
	gm. per 100 ml	*millions*	*per cent*	*cu microns*	*per cent*	*micromicrograms*	*thousands*
None	8 36	9 64	35 5	37 1	23 4	8 6	29 0
	±0 622	±0 577	±1 72	±1 07	±0 52	±0 26	±2 68
Liver	8 77	9 27	35 5	38 6	24 5	9 5	40 3
	±0 604	±0 481	±1 39	±1 19	±0 69	±0 38	±4 90
Fe, Cu, Co	12 86	12 94	47 4	36 8	27 3	10 0	19 1
	±0 614	±0 611	±2 40	±1 49	±0 84	±0 33	±1 55
Stock	16 04	↖ 52	50 4	59 3	31 8	18 9	22 0
	±0 149	±0 194	±0 59	±1 15	±0 47	±0 24	±0 73

Each of the readings is the mean of ten (no treatment and liver) or eight rats with its standard error.

ence in growth was found due to the more rapid growth of the males, as would be expected. A highly significant sex by liver interaction was accounted for in the observation that male rats responded more than did female rats to the liver extract supplement.

Blood Picture—The results of the blood studies are summarized in Table

V. The significance of differences of treatment was determined by the t test.

In comparing the group that received no treatment, that is those rats which received the high zinc diet alone, with the stock rats, it will be observed that hemoglobin and hematocrit values are lower. These differences are highly significant. Although hemoglobin values are lower, the mean red cell count of the untreated group is slightly higher than that of the stock rats. This difference is not significant. The mean cell volume is significantly less (odds 99:1) than normal, which shows that the zinc anemia is microcytic. Also, the mean concentration of hemoglobin per red cell is significantly less (odds 99:1) than normal, and this shows the anemia to be hypochromic. The zinc anemia is, therefore, morphologically classified as microcytic and hypochromic. The leucocyte counts of the untreated group are significantly higher than normal. Apparently, the excess dietary zinc affects the leucogenic as well as the erythrogenic line of cells. Microscopic examinations of the blood smears of the untreated rats confirmed the presence of microcytosis and hypochromia. In addition there was a definite basophilia, as noted in the many red cells which took the hematoxylin stain. Poikilocytes were present, but not numerous.

The addition of liver extract to the high zinc diet did not significantly change the blood picture exhibited by the untreated group except to induce a further rise in the leucocyte counts. This difference was significant. In the previous experiment in which the dietary level of zinc was 0.7 per cent, liver extract maintained the hemoglobin at a level significantly higher than in those rats not fed the liver supplement. However, in this latter experiment, in which the zinc level was 1 per cent, liver extract had no significant effect on the hemoglobin level.

The addition of iron, copper, and cobalt salts to the high zinc diet produced a rise in hemoglobin level, red cell count, and hematocrit. These increases were all highly significant. The red cell counts of the iron-, copper-, and cobalt-supplemented rats were even higher than that of normal rats. This difference was highly significant.

DISCUSSION

The results of this study show that the addition of zinc to an otherwise adequate diet for rats induces at least two symptoms, (1) an anemia that can at least partially be prevented by an additional supplement of copper salts, and (2) subnormal growth that can be partially prevented by an additional supplement of liver extract.

In so far as the anemia is concerned, additional supplements of iron or cobalt salts had no effect, although the combination of iron, copper, and cobalt salts maintained hemoglobin at slightly higher levels than copper alone at the dietary levels of zinc and the supplements used in this study.

It is possible that the feeding of levels of copper higher than that used here would maintain hemoglobin at normal levels. Liver extract fed as a supplement to a 0.7 per cent zinc diet maintained a hemoglobin level higher than that found in rats fed the high zinc diet alone, but it had no significant effect on the level of hemoglobin when the level of zinc was 1 per cent. Liver extract may have affected the course of the anemia through its content of iron, copper, and cobalt. 1 gm. of liver extract furnished an average of 0.11 mg. of iron, 0.03 mg. of copper, and 0.0002 mg. of cobalt. These amounts were considerably less than those furnished by the salts.

A review of the literature revealed another suggestion of a dietary copper-zinc relationship. The condition of enzootic ataxia in lambs in Australia has been associated with a dietary deficiency of copper (Bennetts and Beck (1)). Swayback, a disease similar to the enzootic ataxia of Australia, is wide-spread in Great Britain, and copper supplementation has been found to be an effective preventive (Dunlop et al. (2)), but in contrast to the "ataxia" pastures of Australia the "swayback" pastures of Britain are well supplied with copper (Innes and Shearer (6)). These findings lead Shearer to suggest that some unknown factor in the "swayback" pastures affected the utilization of dietary copper. Such pastures were found to have a high calcium to phosphorus ratio and a high content of zinc and lead, and it was suggested that one of these may affect the absorption of copper.

Iron, copper, and cobalt salts had no effect on the subnormal growth induced by the high zinc diet, but liver extract supplements gave highly significant growth responses. Liver extract, then, furnished some factor or factors that the zinc additions rendered inadequate. In this connection Gross et al. (5) reported that 4 to 6 mg. of zinc chloride fed to rats in addition to a synthetic diet precipitated a deficiency syndrome that could be cured by the additional feeding of calcium pantothenate. Our rats fed the high zinc diets did not show any characteristic symptoms of pantothenic acid deficiency. In a pilot experiment, rats were fed the high zinc diet and in addition a supplement of 100 γ of calcium pantothenate per day. This supplement had no effect on the hemoglobin level or on the rate of growth. Apparently the deficiency induced by the zinc carbonate additions and corrected by the liver extract is not related to pantothenic acid. Further work is required to determine the active fraction in the liver extract.

The significant differences found among replications, i.e. positional effects, in the first study are worthy of further comment. The majority of nutritional experiments are conducted with the assumption, consciously or unconsciously, that positional effects are either not present or that they are of a magnitude that is insignificant. The results reported here showed that positional effects were significant as related to hemoglobin level even

in a small air-conditioned animal room. Even though positional effects
may for the moment be uninterpretable or may not be considered to be of
interest in a particular study, an estimation of their magnitudes would be
justified in the interests of increased precision. In a properly designed
experiment, the effect of position of the animals may be estimated and
removed from the total source of variance, permitting greater precision in
the estimation of the effects of the variables under study. Another reason
for designing experiments in which positional effects can be estimated is
to prevent confounding positional effects with treatment effects. Such
confounding may lead the experimenter to conclusions that treatments were
significantly different when the differences may have been due to the posi-
tion of the animals.

SUMMARY

The feeding of excess zinc to young rats induced a microcytic and hypo-
chromic anemia and subnormal growth. The additional feeding of copper
maintained the hemoglobin at significantly higher levels and a mixture of
iron, copper, and cobalt essentially maintained hemoglobin at normal
levels, although iron or cobalt supplements alone had no effect. Iron,
copper, and cobalt had no effect on the subnormal growth of zinc-fed rats,
but supplements of a liver extract produced a highly significant growth
response. The liver activity is apparently not due to pantothenic acid.

Studies such as this point to the possible occurrence of relative deficiencies
in addition to absolute deficiencies in the field; that is, animals may be
deficient in one mineral relative to an excess of some other.

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STUDIES ON CHOLINESTERASE*

II. ENZYME ACTIVITY AND VOLTAGE OF THE ACTION POTENTIAL IN ELECTRIC TISSUE

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Nerve impulses are conducted with great velocity. In mammalian nerve the speed of conduction may be as high as 100 meters per second. The energy required for the propagation of the impulse is extremely small, being less than 10^{-7} gm. calorie per gm. and impulse. It is impossible to determine directly the chemical cell constituents involved in such a rapid process, especially since they occur in quantities of the order of magnitude of fractions of a microgram. However, the enzymes catalyzing the chemical reactions connected with conduction are relatively stable, and, by their study, valuable information as to the biochemistry of this process may be obtained. Investigations on cholinesterase, an esterase specific for acetylcholine, have established that the enzyme is highly concentrated in nerve tissue. The concentration is sufficiently high to permit the assumption that the rate of acetylcholine metabolism may parallel that of the electric changes observed during the conduction of the nerve impulses. The amount of acetylcholine which may be hydrolyzed per millisecond, *i.e.* during the passage of an impulse, may cover a considerable surface area. The enzyme, moreover, is localized exclusively in the neuronal surface, where the bioelectrical phenomena occur (1).

These facts make possible the assumption that acetylcholine is directly connected with the conduction of the nerve impulse, that is with the nerve action potential. However, studies of enzyme activities *in vitro* are insufficient for the interpretation of the precise rôle of a chemical compound in a cellular mechanism. They have to be correlated with a process occurring in the living cell. Such a correlation could be established in experiments on the electric organ of fish. It was found, in 1937, that the electric organ of *Torpedo marmorata* may hydrolyze 2 to 3 gm. of acetylcholine per gm. per hour. This is the first recorded evidence for a possible relationship between cholinesterase and the electric discharge. A similarly high concentration

of the enzyme was found in the electric organ of *Electrophorus electricus*. This extraordinary enzyme concentration appears particularly significant in view of the low protein content of these organs (2 per cent). In the weak electric organ of the common ray the concentration of the enzyme is low.

The voltage of the discharge in these organs depends upon the number of electric plates. Each plate has a voltage of the order of 0.1 volt, which is the same order of magnitude as that found in ordinary nerves. The discharge is basically identical with the ordinary nerve action potential, the only difference being the arrangement in series (2). The greater the number of plates in series, the higher the voltage. If in the three species mentioned voltage per cm., number of plates per cm., and concentration of cholinesterase are compared, a parallelism becomes obvious (3).

A more quantitative relationship between voltage and cholinesterase has been established in experiments on the electric organ of *Electrophorus electricus*. In this organ the number of plates and the voltage per cm. decrease from the head to the caudal end of the organ, and the cholinesterase concentration decreases in similar proportion (4, 5). Also, at corresponding points along the organs of different specimens, wide variations are found in the voltage and the number of plates per cm., especially when the specimens are of different size. Even if measured on different specimens, the enzyme activity and voltage appear to have the same ratio.

A parallelism between the activity of a specific enzyme and a physical event recorded on the intact animal with a highly sensitive method (cathode ray oscillograph) is always important. In order to examine how quantitative this parallelism may be, it appeared necessary to test a larger number of specimens of varying sizes and at different areas of the same organ. The data obtained are presented in this paper.

Methods

Both the electrical and the chemical methods used were the same as described previously (5). The distance between the two electrodes was always 5 cm., whereas in earlier experiments it was 5 cm. for small and 10 cm. for large specimens. The voltages indicated in Tables I to IV are always the maximum voltages obtained on an open circuit. Tetanic discharges of the fish were carefully avoided, as this soon resulted in fatigue with consequent decrease in voltage. If this were the case, the fish was allowed to rest in the tank for a considerable length of time until completely recovered or another specimen was used.

The grinding of the tissue (100 to 200 mg. each) was always carried out mechanically by a homogenizer with the addition of silicate powder. The importance of this addition for obtaining a homogenized suspension with

complete destruction of the cells has been previously emphasized. On several occasions, two determinations were made with the same suspension. Excellent agreement was always obtained, indicating that the suspensions were really homogeneous.

Results

The specimens available ranged from 44 to 133.5 cm. in length. There are two pairs of electric organs in *Electrophorus electricus*. One pair with high voltage, the so called large electric organs, is found all along the anterior part of the fish; the other pair with low voltage, the bundles of Sachs, is located in the posterior part. The highest voltage per cm. was 22 volts, which was obtained in a specimen of 48.5 cm. in length in the portion close to the head end of the large electric organ. The lowest voltage obtained was 0.55 volt per cm. in the bundles of Sachs of a specimen of 114.5 cm. in length.

Tables I to IV give all the data obtained. In Table I are recorded the experiments on the large electric organs with small specimens; in Table II those with medium sized and larger specimens. Electric measurements were made on two or three segments of each specimen and one or two samples were taken from each point recorded for chemical analysis. In experiments on four specimens, five samples were used for chemical analysis from each point recorded electrically .in order to obtain a more complete picture of the possible range of variations of the enzyme concentration in a given segment. These data are summarized in Table III. Since the suspensions were really homogeneous, as the control determinations mentioned above had shown, a lack of agreement of duplicate and quintuplicate determinations on various parts of a single segment gives a measure of the heterogeneity of the tissue. This heterogeneity is measured by a standard deviation on the duplicate or quintuplicate determinations of 19 units of concentration and is about 9 per cent of the average concentration. This is easily conceivable, since the samples taken from the same segment will vary to a certain degree in their histological composition (*i.e.*, the amount of connective tissue, or, more generally speaking, the ratio of inactive tissue to active tissue may not always be exactly the same).

In Table IV a few data are given of determinations on the bundles of Sachs. They are rather difficult to carry out on living fish since at that part where the bundles of Sachs are located there is usually a large layer of subcutaneous fat and in trying to cut out the electric tissue the swim bladder is easily punctured, an event fatal to the fish. Two data were used from earlier measurements.

In the last column of Tables I to IV, the quotient of cholinesterase over voltage (ChE/V) is recorded. 55 such quotients have been obtained.

TABLE I

Relationship between Concentration of Cholinesterase and Voltage of Action Potential
(ChE/V)

The large electric organs of small specimens were used. L = length of the fish in cm.; D = distance of the electrodes from the anterior end of the strong electric organ in cm.; V = maximum voltage per cm. in volts; QChE = mg. of acetylcholine split by 100 mg. of fresh tissue in 60 minutes; s = data obtained from a single piece of tissue; a = average from two or more.

L	D	V	QChE s	QChE a	$\frac{ChE}{V}$
44.0	0.5- 5.5	15	461		30.7
46.2	0.5- 5.5	18	389		21.6
	8.5-13.5	14	444		31.7
48.5	0.5- 5.5	22	392		17.8
	9.0-14.0	16	242		15.1
54.5	0.5- 5.5	19	325		17.1
	12.5-17.5	13	278		21.4
54.5	0.5- 5.5	21	451		21.6
	10.5-15.5	15	329		21.9
64.0	1.0- 6.0	20	435, 396	416	20.8
	10.0-15.0	16	253, 270	262	16.4
	18.0-23.0	8.6	170		19.8
66.5	0.5- 5.5	15	368		24.5
	10.5-15.5	13	311, 292	302	23.2
71.0	0.5- 5.5	16	248		15.5
	10.0-15.0	14	272, 226	249	17.8
	18.0-23.0	9.5	215, 193	204	21.5
75.0	1.0- 6.0	16	308, 316	312	19.5
	11.0-16.0	12	290, 284	287	23.9
	21.0-26.0	6.9	212, 182	197	28.5
77.0	1.0- 6.0	17	293, 278	286	16.8
	10.0-15.0	13	267, 251	259	19.9
	20.5-25.5	8.9	211, 183	197	22.1

When two or more samples were taken from one segment, the average obtained was used for the calculation of the quotient.   The average value of

the 55 determinations is 20.7 with a standard deviation of ± 0.7. This indicates that the true average ratio probably lies within 20.7 ± 1.4. The

TABLE II

Relationship between Concentration of Cholinesterase and Voltage of Action Potential (ChE/V)

The large electric organs of medium sized and larger specimens were used. The abbreviations are as in Table I.

L	D	V	QChE		$\frac{ChE}{V}$
			s	a	
92.5	26.0–31.0	5.7	115		20.2
93.0	2.0– 7.0	14	242		17.3
	18.0–23.0	7.8	186 164	175	22.4
	27.5–32.5	4.5	110		24.5
97.5	2.0– 7.0	15	371 332	352	23.5
	16.0–21.0	11	233 265	249	22.6
	27.0–32.0	8.2	246 284	265	32.3
98.5	26.5–31.5	7.8	139		17.8
103.0	1.0– 6.0	11	331 308	320	29.1
	15.0–20.0	8.6	219 217	218	25.3
	29.0–34.0	4.5	141 129	135	30.0
113.0	27.0–32.0	6.6	123		18.7
114.5	34.5–39.5	7.7	151		19.6
123.5	1.0– 6.0	8.3	132 179	156	18.8
	18.0–23.0	7.0	113 96	105	15.0
	28.0–33.0	5.3	72		13.6
131.0	31.0–36.0	8.0	158		19.8
133.5	2.0– 7.0	12	185 154	170	14.2
	23.0–28.0	10	145 122	134	13.4
	38.0–43.0	5.4	75 80	78	14.5

55 quotients are quite variable, however, as indicated by the standard deviation of ± 5.1 for a single measurement. Indeed it may be observed from Tables I to IV that the ratios vary from 13.4 to 35.3, although most of them

vary within 5 from the average. This is good uniformity for a quotient relating physical and chemical data.

<div align="center">TABLE III</div>

Relationship between Concentration of Cholinesterase and Voltage of Action Potential
(ChE/V)

In this series five samples were taken from each area. The abbreviations are as in Table I.

L	D	V	QChE s	QChE a	$\frac{ChE}{V}$	D	V	QChE s	QChE a	$\frac{ChE}{V}$
72.0	0.5–5.5	15	245			15.5–20.5	13	158		
			195					203		
			228					192		
			268					211		
			201	227	15.1			184	190	14.6
78.5	0.5–5.5	17	266			20.5–25.5	9 6	160		
			302					214		
			290					197		
			285					160		
			286	286	16 8			170	180 ·	18.8
88.5	1.0–6.0	14	197			21 0–26.0	6.3	138		
			189					115		
			190					125		
			163					132		
			206	189	13.5			134	129	20 5
118.5	1.0–6.0	9.8	171			31.0–36.0	4.0	84		
			152					64		
			189					68		
			150					86		
			157	164	16.8			69	74	18 5

<div align="center">TABLE IV</div>

Relationship between Concentration of Cholinesterase and Voltage of Action Potential
(ChE/V) in Bundles of Sachs

The abbreviations are as in Table I.

L	D	V	QChE	$\frac{ChE}{V}$
76	42.0–47.0	0.62	21.9	35.3
105	45.0–50.0	0.91	21	23.1
105	60.0–70.0	0.90	23.2	25.8
114.5	66.5–71.5	0.55	10.5	19.1

The correlation between the two variables may best be seen from Fig. 1, where voltage is plotted against QChE. From all 55 determinations calcu-

lated according to the method of least squares $y = 13.2 + 18.9x$, where y $= Q$ChE and $x =$ voltage. The relation indicated by these values is significant statistically. Since, however, 13.2 is not significantly different from 0, it may be neglected in this instance and assumed that the real line goes through the 0 point (Fig. 1). In that case, $y = 19.9x$, the line again being calculated by the method of least squares. It may be noted that 19.9 is close to the average ratio of 20.7.

If the data are arranged in the order of decreasing voltage and split into four equal sets of pieces having voltages per cm. of 22 to 15, 15 to 12, 11 to 8, 7.8 to 4.0, the corresponding average values of ChE/V would be 19.4,
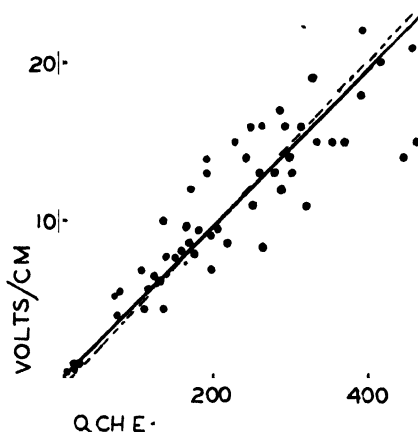
FIG. 1. Correlation between voltage and cholinesterase activity. The voltage per cm. is plotted against the enzyme concentration. The dotted line is calculated from the data obtained with the method of least squares; the fully drawn line calculated on the assumption that the line goes through the 0 point.

20.1, 21.4, and 20.3. Thus the deviations of ChE/V seem to be only small and possibly completely random.

DISCUSSION

The relationship between voltage and cholinesterase activity in electric tissue, suggested by previous observations, has been studied this time on the basis of a great number of measurements covering a wide range of variations. Thus, a statistical evaluation becomes possible. The average quotient of ChE/V may be assumed to be a very reliable value, since its standard deviation was only 0.7 or 3.3 per cent.

Of particular importance is the fact that the line correlating the two variables apparently goes through 0. This indicates a direct proportionality. The results appear significant and consistent with the concept that

the physical and chemical processes recorded are directly associated and consequently interdependent.

There is one exception. In a previous paper (5) three observations made on the caudal end of the large electric organs were reported in which the voltages per cm. were of the order of 1; the enzyme concentration decreased caudally, but the ratio ChE/V at this particular point was several times higher than elsewhere. At the caudal end of the main electric organ the situation is, however, rather complex and other quantities characteristic of the tissue behave differently (Cox, Coates, and Brown (6)). The organ has tapered down to a thin layer and forms only a small fraction of the cross-sectional area. It would hardly be surprising if a deviation of the otherwise constant ratio ChE/V were found there. The data in the previous paper are too few and other kinds of measurements would be necessary for any attempt of interpretation. The observations of the present paper clearly establish the uniformity of the ratio in nearly the whole large electric organ as well as in the bundles of Sachs. It is obvious that the relation between enzyme activity and the electric manifestations cannot be expected to remain strictly proportional if other factors change the conditions, as for instance the ratio of active to inactive tissue.

The meaning of the relationship between the enzyme activity and the voltage of the action potential observed has been repeatedly discussed (1, 5) and need not be considered here. It may suffice to say that such a parallelism between the voltage and the enzyme concentration may be pictured in either of two ways. The compound metabolized by the enzyme may generate electromotive force directly, or alternatively it may lower the resistance. No conclusive evidence is available from experiments on nerve that electromotive force is generated. Since recent investigations (6) have shown that during the passage of the impulse the resistance drops considerably, the best interpretation of the relationship observed seems to be at present that the ester acts in such a way as to decrease the resistance.

It should be noted that the esterase activity measured is exclusively that of the specific cholinesterase (7). As recently shown, the enzyme even when purified to a high degree still shows the same pattern as that of the homogenized suspension of electric tissue. Consequently, the enzyme activity may be used as an indication of the potential rate of the metabolism of acetylcholine and the correlation found may be referred to a parallelism between acetylcholine metabolism and voltage (1).

The relation between acetylcholine and the nerve action potential which results from the experiments on the electric organ has recently found further support in two other lines of investigations. One is based on the energy transformations occurring during the action potential. It has been found that energy-rich phosphate bonds are adequate in accounting for the total

electric energy released by the action potential (8). Consequently, if the release of acetylcholine is the primary "excitatory disturbance" (Keith Lucas), responsible for the nerve action potential, energy-rich phosphate bonds should be used for the synthesis of the ester. This is actually the case: an enzyme, choline acetylase, is present in brain and peripheral nerve which in the presence of adenosine triphosphate forms acetylcholine in anaerobic condition at a high rate (9–13). This fact is consistent with the conclusion that the energy of the primary recovery process after the passage of the impulse is used for the resynthesis of the ester removed during activity.

Another relationship between nerve action potential and acetylcholine has been established by experiments on the giant axon and the fin nerve of squid (Bullock, Nachmansohn, and Rothenberg (14)). Acetylcholine is a quaternary ammonium salt and cannot permeate the lipid membrane surrounding the axon. When it is applied externally, its effect is limited to nerve endings which do not have a myelin sheath. But, if the cholinesterase is inactivated by drugs which may permeate the lipid membranes and are strong inhibitors of the enzyme, like eserine or strychnine, acetylcholine released internally cannot be removed; a state of depolarization persists and the nerve action potential is abolished. This process is easily reversible, as should be expected from the reversibility of the enzyme inhibition.

Each of these observations considered separately constitutes a strong support for the assumption of a close connection of acetylcholine with conduction of the nerve impulse. But combined, the value of each of them is potentiated and the concept that acetylcholine plays an essential rôle in the nerve action potential appears highly probable.

SUMMARY

The activity of cholinesterase and the voltage of the action potential have been measured simultaneously in the electric organ of a number of specimens of *Electrophorus electricus* and at different regions of the organ of each specimen. Since the voltage per cm. varies at different points of the same specimen and depends also on the size, a considerable range of variations from 0.5 to 22.0 volts per cm. has been covered. The results confirm and extend previous findings indicating a close parallelism between the activity of the enzyme and the voltage of the action potential.

In 55 experiments the average of the quotient cholinesterase over voltage was found to be 20.7 with a standard deviation of ±0.7. The standard deviation of a single measurement of the quotient is ±5.1.

If the voltage is plotted against the cholinesterase activity, the line which correlates the two variables passes apparently through the 0 point. This

supports the assumption of a direct proportionality between physical and chemical events measured.   Combined with other observations referred to in the discussion, the direct connection of acetylcholine with the nerve action potential and nerve conductivity becomes highly probable.

We are greatly indebted to Dr. R. T. Cox and Dr. M. Vertner Brown for their help in the electric recordings, and to Dr. John W. Fertig for his help in the statistical analysis of the data.

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THE SYNTHESIS AND THE ENZYMATIC DEGRADATION OF
l-TYROSYL-l-LYSYL-l-GLUTAMYL-l-TYROSINE

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The formation of angiotonin from the serum protein fraction α_2-globulin and renin (1, 2) as well as its inactivation by proteolytic enzymes suggests that this pressor substance contains peptide linkages. Preliminary studies and interpretations (3) of the action of crystalline proteolytic enzymes led to the conclusion that angiotonin contains groupings of which the simplest representation is afforded by

$$\underset{\text{Aromatic}}{NH_2-CH}-CO-NH-\underset{\text{Basic}}{CH}-CO-NH-\underset{\text{Acid}}{CH}-CO-NH-\underset{\text{Aromatic}}{CH}-COOH$$
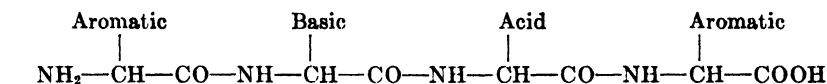
The object of this investigation was to prepare a tetrapeptide similar in structure which would not only be of interest for the study of the kinetics of enzyme action but might serve as a model for degradation experiments on naturally occurring polypeptides. A comparison of the rates of hydrolysis of such a synthetic tetrapeptide with angiotonin might give some valuable information concerning the structure of this pressor substance.

Since the rate of hydrolysis of a polypeptide by a well defined proteolytic enzyme appears to be a function of the structure of the substrate as well as of the general nature of the enzyme, a comparison of the rate of hydrolysis might be used for identification purposes. A compound containing amino acids in the arrangement outlined above should be susceptible to hydrolysis by carboxypeptidase, trypsin, chymotrypsin, pepsin, and, if there exists any structural similarity to angiotonin, the rates of hydrolysis by these enzymes should be comparable.

The amino acids tyrosine, lysine, and glutamic acid were selected for the synthesis, because a number of necessary intermediates and methods for their syntheses are known. The synthesis herein reported consists of a condensation of two dipeptides whose functional groups were protected, followed by removal of the protecting groups by standard methods.

The first dipeptide was prepared by a condensation of O-acetyl-N-car-

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bobenzoxy-*l*-tyrosyl chloride (I) with ε-carbobenzoxy-*l*-lysine methyl ester (II). Minor variations in the procedure resulted in two distinctly different condensation products, one melting at 156° and the other at 138°. Both substances yielded the same hydrazide on reaction with hydrazine hydrate. Since there is no possibility of a racemization, a structural difference other than asymmetry must be assumed to explain this phenomenon.

The condensation of O-acetyl-N-carbobenzoxytyrosyl chloride and the ε-carbobenzoxylysine methyl ester is carried out in such a way that the acid chloride is added to an excess of the basic ester, so that during the whole experiment the medium is slightly alkaline. It therefore offers the possibility for hydrolysis of very susceptible ester linkages such as the acetyl group on the phenolic hydroxyl of the tyrosine, leaving the carboxylic

TABLE I

Comparison of Acetyl Determinations on Carbobenzoxy Compounds with and without Acetyl Groups

Compound	Formula	Per cent acetyl ($COCH_3$)	
		Calculated	Found
N-Carbobenzoxy-*l*-tyrosine ethyl ester	$C_{19}H_{21}O_5N_3$	None	2.46
N-Carbobenzoxy-*l*-tyrosine	$C_{17}H_{17}O_5N$	"	2.06
O-Acetyl-N-carbobenzoxy-*l*-tyrosine	$C_{21}H_{23}O_6N$	11.08	12.20
O-Acetyl-N-carbobenzoxy-*l*-tyrosyl-N-carbobenzoxy-*l*-lysine methyl ester, m.p. 138°..	$C_{34}H_{39}O_9N_3$	6.80	8.06
N-Carbobenzoxy-*l*-tyrosyl-N-carbobenzoxy-*l*-lysine methyl ester, m.p. 156°	$C_{32}H_{36}O_8N_3$	None	2.84

ester of the lysine portion intact. The conversion of the ester (III) to the hydrazide is carried out in an even stronger alkaline medium, thus converting both the acetylated as well as the deacetylated compound into the same hydrazide.

The compound with a melting point of 156° gave an analytical value of 8.06 per cent of acetyl; the compound with a melting point of 138° gave 2.84 per cent. The theoretical values for the acetylated and deacetylated esters are 6.80 and 0.00 respectively. The suspicion that the discrepancies might be due to the presence of the carbobenzoxy group was confirmed by acetyl analyses on model substances (Table I).

An attempt was made to prepare the other moiety of the desired tetrapeptide by esterifying carbobenzoxy-*l*-glutamyl-*l*-tyrosine monoethyl ester, followed by removal of the carbobenzoxy group. The esterification proceeded satisfactorily and the expected carbobenzoxy diethyl ester was isolated in good yield. On treatment with palladium and hydrogen in

OAc

(II)

NHCbzo

CH_2

$(CH_2)_3$

CH_2

NH—CH—CO—Cl H_2N—CH—COOMe

COOH

CH_2

CH_2

OH

(V)

CH_2

Cbzo—NH—CH—CO—NH—CH—COOEt

a) R = H

= $COCH_3$

OR

NHCbzo

CH_2

$(CH_2)_3$

CH_2

Cbzo—NH—CH—CO—NH—CH—COOMe

COOEt

CH_2

CH_2

OH

(VI)

CH_2

Cbzo—NH—CH—CO—NH—CH—COOEt

Hydrazide (IV)   Free diester (VII)

OH

NHCbzo

CH_2

$(CH_2)_3$

COOEt

CH_2

CH_2

OH

(VIII)

CH_2

CH_2

)zo—NH—CH—CO—NH—CH—CO—NH—CH—CO—NH—CH—COOEt

OH

NH_2

CH_2

$(CH_2)_3$

COOH

CH_2

CH_2

OH

(IX)

CH_2

CH_2

NH_2—CH—CO—NH—CH—CO—NH—CH—CO—NH—CH—COOH

l-Tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine

alcoholic solution, an ill defined, though crystalline substance was obtained. Analytical data suggested it to be a mixture of l-glutamyl-l-tyrosine mono- and diethyl esters. This procedure was therefore discarded in favor of the method of Fruton and Bergmann (4) who prepared the diethyl ester as an intermediate in the synthesis of glycyl-l-glutamyl-l-tyrosine. The ester was not isolated in crystalline form.

The hydrazide of the tyrosyllysine moiety, when allowed to react with nitrous acid in acetic acid solution, gave a crystalline azide which was immediately treated with l-glutamyl-l-tyrosine diethyl ester (VII) in ethyl acetate solution. Although the crude condensation product was obtained in good yield, an analytically pure material was difficult to prepare. The purity of this tetrapeptide derivative appeared to be particularly important, because the anticipated removal of the protecting groups would yield a water-soluble compound whose molecular weight could not be determined by the conventional micromethods. Upon repeated recrystallization from various solvent mixtures and finally from pure ethyl alcohol the compound was obtained in pure form; its solubility in organic solvents and camphor rendered it suitable for the determination of its molecular weight by the Rast method (5). These values were in good agreement with theory. This, together with the correct analytical figures for carbon, hydrogen, and nitrogen as well as ethoxyl and the absence of amino nitrogen may be taken as evidence that condensation to the tetrapeptide had taken place.

Saponification of VIII under mild conditions followed by removal of the two carbobenzoxy groups by catalytic hydrogenation with palladium black proceeded without difficulty and gave the desired l-tyrosyl-l-lysyl-l-glutamyl-l-tyrosine as the tetrahydrate. The final compound was optically active. It did not affect the arterial blood pressure of a pithed cat when injected in doses of 20 mg. per kilo.

Since this compound contains two aromatic, one basic and one dicarboxylic amino acid, it should be sensitive to hydrolysis by a number of proteolytic enzymes. The structural detail of a substrate necessary to render it susceptible to hydrolysis by proteolytic enzymes has been carefully investigated by Bergmann and his associates. The indispensable groups of substrates for carboxypeptidases according to Bergmann (6) are

$$\begin{array}{c} R \\ | \\ \ldots\text{---NH---CH---COOH} \\ (X) \end{array}$$
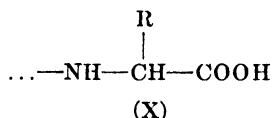
where R is a hydroxybenzyl or benzyl radical. The tetrapeptide which we have synthesized contains such an arrangement (Structure A, Fig. 1) and hence should be attacked by carboxypeptidase and the hydrolysis should
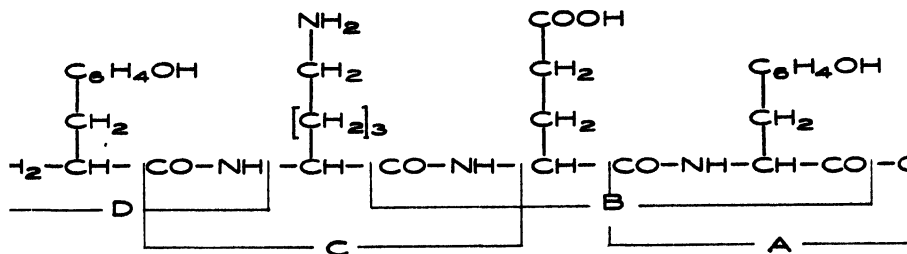
FIG. 1. Structural formula for *l*-tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine illustrating the necessary arrangements for hydrolysis by proteolytic enzymes: *A*, carboxypeptidase; *B*, pepsin; *C*, trypsin; *D*, chymotrypsin.
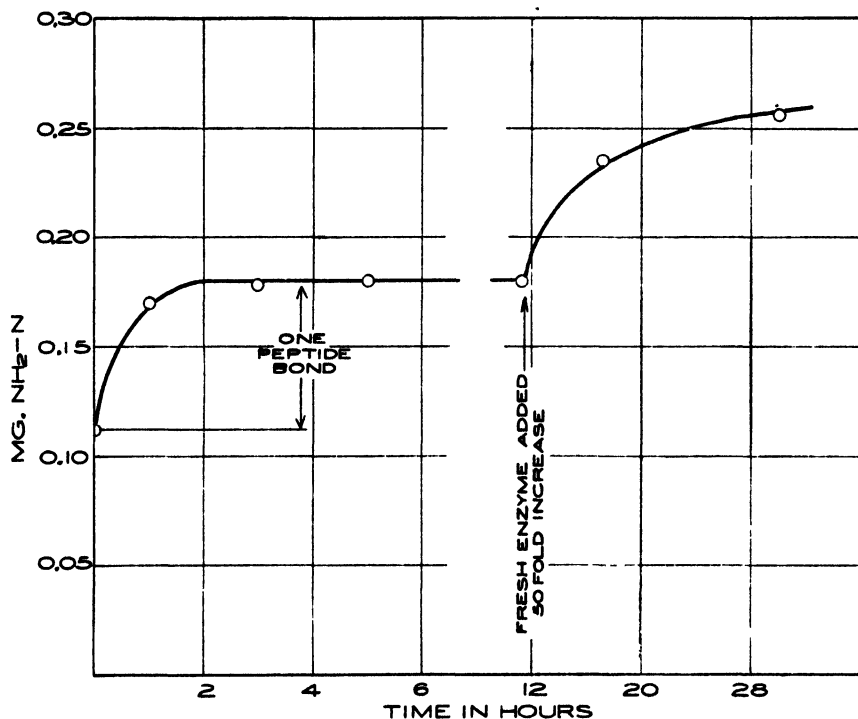
FIG. 2. The course of hydrolysis of *l*-tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine by carboxypeptidase. The enzyme concentration was 0.0067 mg. of N per cc. of test solution; the pH was maintained at 7.8 by means of 0.1 M phosphate buffer. Incubation temperature 36°.

proceed with the liberation of 1 molecule of tyrosine. The resulting tetrapeptide, with a glutamic acid residue in terminal position, no longer contains the requisite groups in the backbone and may be expected to resist

further hydrolysis by this enzyme. Although Hofmann and Bergmann (7) found that a compound containing glutamic acid in terminal position can be hydrolyzed by carboxypeptidase, it should be noted that an exceedingly high concentration of the enzyme was necessary in order to hydrolyze carbobenzoxyglycyl-*l*-glutamic acid (7). These authors employed almost 100 times as much enzyme for the hydrolysis of carbobenzoxyglycyl-*l*-glutamic acid as we have used in our experiments. Inspection of Fig. 2 will reveal that exactly 1 mole of amino acid nitrogen has been liberated after 3 hours incubation of the tetrapeptide with carboxypeptidase with no further increase during the ensuing 9 hours. The concentration of carboxypeptidase was thereupon increased 50-fold, when a significant rise in amino nitrogen was observed, indicating that hydrolysis of the tripeptide had taken place, probably with formation of tyrosine, lysine, and glutamic acid. The hydrolysis of tyrosyllysylglutamyltyrosine by crystalline carboxypeptidase thus proceeded as expected and fully confirmed the specificity requirements for this enzyme, as advanced by Bergmann (6).

The specificity requirements for pepsin (6) call for the following arrangement of amino acids in the substrate molecule

$$R_1 \qquad\qquad R_2$$
$$| \qquad\qquad |$$
$$\text{—NH—CH—CO—NH—CH}\ldots$$
$$(XI)$$
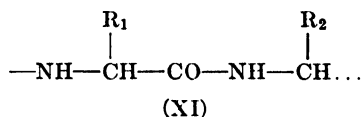
where R_2 must be a benzyl- or hydroxybenzyl radical and R_1 an inert or preferably dicarboxylic amino acid residue such as glutamic acid. Since our tetrapeptide contains such atomic groups in its backbone and side chain (Structure B, Fig. 1), it should be sensitive to peptic digestion, and this is borne out by the experimental data reproduced in Fig. 3. The increase in amino nitrogen on incubation with pepsin corresponds to one peptide bond with no further increase on prolonged digestion. It must be concluded that pepsin attacks the substrate on the same sensitive peptide bond as does carboxypeptidase; hence both liberate 1 mole of tyrosine.

Contrary to our expectations, the residual tripeptide, tyrosyllysylglutamic acid, was entirely resistant to tryptic action, even in exceedingly high concentrations of the enzyme. The tripeptide contains the requisite groups (Structure C, Fig. 1) for typical trypsin substrates (6); *i.e.*, the amino acid residue R in XII should be a lysyl

$$R$$
$$|$$
$$\text{—CO—NH—CH—CO—NH}\ldots$$
$$(XII)$$
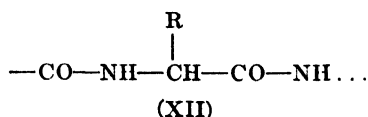
or arginyl radical with its amino and carboxyl groups involved in a peptide linkage. Since the concentration of both enzymes, pepsin and trypsin, was
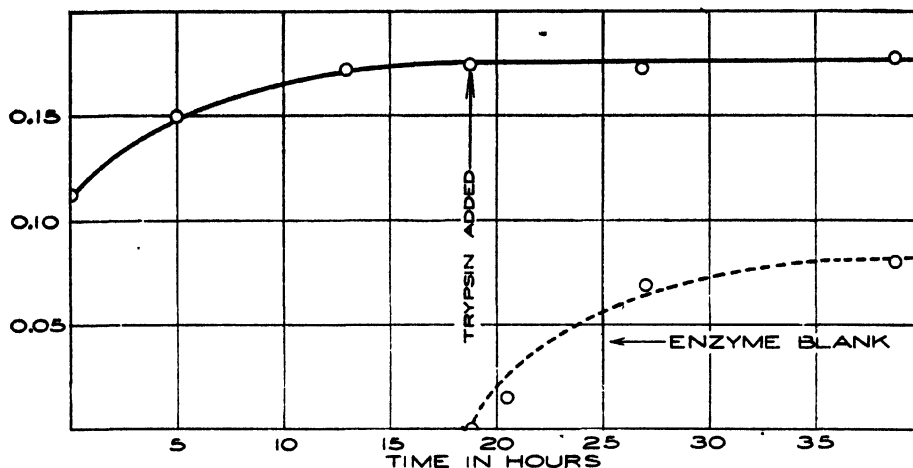
FIG. 3. The course of hydrolysis of *l*-tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine by pepsin, followed by trypsin. The enzyme concentration was 0.495 mg. of pepsin N and 0.094 mg of trypsin N per cc. of test solution. During peptic digestion the pH was maintained at 3.0 by means of citrate buffer and adjusted to pH 7.8 after the addition of trypsin. The enzyme blank was determined on a solution of pepsin and trypsin in the same concentration as the test solution and maintained at pH 7.8. Incubation temperature 36°.
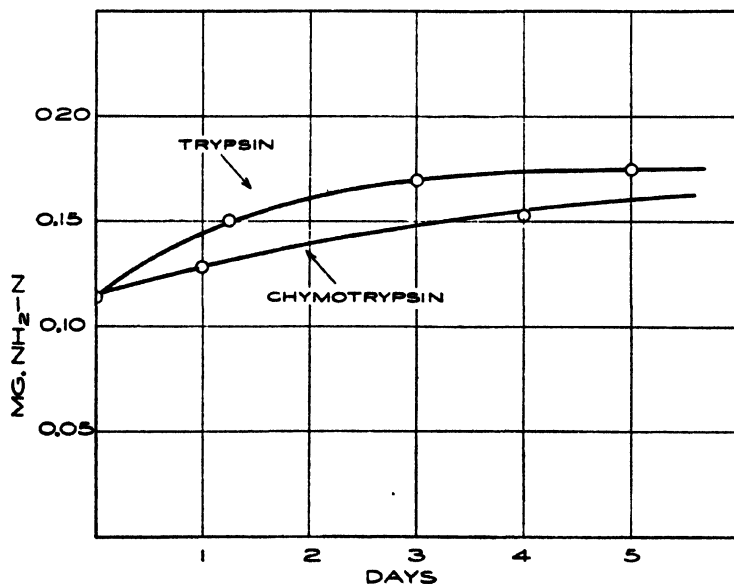
FIG. 4. The course of hydrolysis of *l*-tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine by trypsin and chymotrypsin. The enzyme concentration was 0.094 mg. of trypsin and 0.233 mg. of chymotrypsin N per cc. of test solution; pH 7.8 and incubation temperature 36°.

55

quite high, the inertness of the *tripeptide* to tryptic hydrolysis (*i.e.*, after peptic digestion of the tetrapeptide) might conceivably be attributed to an inhibiting effect of digestion products resulting from the action of trypsin on pepsin. That a number of polypeptides are formed in appreciable amount is indicated by the constantly increasing enzyme blank (Fig. 3) after the addition of trypsin to the tripeptide-tyrosine-pepsin mixture. In order to avoid such complications the action of crystalline trypsin on the tetrapeptide, *i.e.* without previous pepsin digestion, was investigated (Fig. 4). Although the rate of hydrolysis was quite slow compared to carboxypeptidase or pepsin, this substrate was hydrolyzed at a speed comparable to other typical trypsin substrates such as benzoyl-*l*-lysinamide (6) and has the advantage that the course of the hydrolysis can be followed by amino nitrogen determinations rather than titration of the liberated carboxyl groups.

Fruton and Bergmann (8) investigated the action of chymotrypsin on a number of synthetic substrates and found it to exhibit the phenomenon of multiple specificity. Chymotrypsin can act as a carbonyl proteinase (endopeptidase) or aminopeptidase (exopeptidase) requiring an aromatic amino acid whose carboxyl or amino group or both must be involved in a peptide linkage. The necessary structural detail is given by either XIII or XIV

$$
\begin{array}{cc}
\text{R} & \text{R} \\
| & | \\
\text{NH}_2\text{---CH---CO---NH} .. & \text{---NH---CH---CO---NH} \\
\text{(XIII)} & \text{(XIV)}
\end{array}
$$
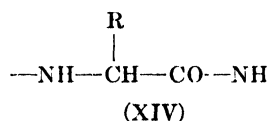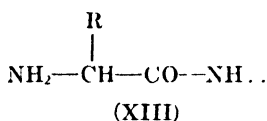
where R must be a benzyl or hydroxybenzyl radical. Since tyrosyl-*l*-lysyl-*l*-glutamyl-*l*-tyrosine contains one such grouping (Structure D, Fig. 1), chymotrypsin must exert at least its aminopeptidase effect; *i.e.*, liberate 1 mole of tyrosine from 1 mole of the tetrapeptide. The increase in amino nitrogen with time obtained by chymotryptic digestion of the tetrapeptide is reproduced in Fig. 4. As anticipated, 1 mole of amino nitrogen was liberated during digestion. This amount of amino nitrogen must be attributed to tyrosine if the enzyme action is due to exopeptidase activity. If, on the other hand, the enzyme should exhibit endopeptidase activity, two dipeptides would be formed. An α-aminocarboxyl determination according to the ninhydrin method of Van Slyke, Dillon, Mac-Fadyen, and Hamilton (9) after 96 hours digestion gave a value which corresponds to 97 per cent of the amino nitrogen liberated during the reaction. This may therefore serve as evidence that chymotrypsin had hydrolyzed the first peptide bond (between the tyrosine and lysine amino acid residues) and hence had acted as an exopeptidase.

The hydrolysis of the tetrapeptide by the four crystalline enzymes conforms with the concept that all proteolytic enzymes hydrolyze the peptide linkage but that it must be in a specific and proper environment of amino acid residues. The inactivation of angiotonin by proteolytic enzymes suggested in a qualitative way that it contains at least three peptide linkages in an environment of amino acid residues similar to l-tyrosyl-l-lysyl-l-glutamyl-l-tyrosine. It was hoped that a quantitative study of the action of crystalline enzymes on this tetrapeptide might yield some additional information regarding the "environment" of the peptide linkages in angiotonin.

TABLE II

Comparison of Action of Crystalline Proteolytic Enzymes on Angiotonin and l-Tyrosyl-l-lysyl-l-glutamyl-l-tyrosine

	Carboxypeptidase	Chymotrypsin	Trypsin	Pepsin
Angiotonin				
Protein N per cc. test solution, mg.	0.0060	0 0097	0.0018	0 053
pH	7.4	7 1	7.2	4 5
K^*	0.030	0.036	0.016	0.042
$C^\dagger$	5.00	3.7	86.0	0 87
l-Tyrosyl-l-lysyl-l-glutamyl-l-tyrosine				
Protein N per cc. test solution, mg.	0.0067	0.233	0.094	0 495
pH	7.8	7 8	7 8	3 0
K^*	0 0016	0.000104	0 000303	0 00182
$C^\dagger$	0.24	0.00045	0 00323	0.036

$$K = \frac{2.3}{t} \log \frac{100}{100 - \% \text{ hydrolysis}}; \quad t = \text{incubation time in minutes.}$$

$$\dagger\ C = \frac{K}{\text{mg. protein N per cc. test solution}}.$$

Reaction constants and proteolytic coefficients were calculated from the various data of Figs. 2 to 4 and compared to the corresponding values previously reported (3) (Table II). A strict comparison between the two sets of data is not possible, since the proteolytic coefficients for both substrates and the four enzymes should be known over the whole pH range. Nevertheless, a few tentative conclusions can be drawn. Although the proteolytic coefficients for pepsin and carboxypeptidase are more than 10 times greater for angiotonin than for the tetrapeptide, this does not necessarily indicate a difference in structure of the substrate. The values for

the action of trypsin and chymotrypsin on these two substrates are sufficiently wide to suggest a fundamental difference. The extreme sensitivity of angiotonin to the latter two enzymes must be due to a structural arrangement which had not as yet been realized in any synthetic substrate. Particularly the peptide linkage in angiotonin which is sensitive to tryptic action must be distinctly different from Structure C, Fig. 1. Since the substitution of arginine for lysine would only slightly more than double the value of the proteolytic coefficient (6), this sensitivity of angiotonin to trypsin possibly might be attributed to a new basic amino acid. Another possibility is that specificity requirements for trypsin have been too narrowly defined and must be broadened.

The data on chymotrypsin do not lend themselves to such a comparison, since the proteolytic coefficient recorded in Table I for this enzyme refers only to its exopeptidase activity. It is conceivable, though unlikely (3), that chymotrypsin acts on angiotonin as an endopeptidase and an interpretation of the data on this enzyme should therefore be postponed until a suitable substrate becomes available.

EXPERIMENTAL

O-Acetyl-N-carbobenzoxy-l-tyrosyl Chloride (I)—This compound was prepared from O-acetyl-N-carbobenzoxy-*l*-tyrosine and phosphorus pentachloride according the method of Bergmann, Zervas, Salzmann, and Schleich (10).

ε-Carbobenzoxy-l-lysine Methyl Ester (II)—To a solution of 4.5 gm. of ε-carbobenzoxy-*l*-lysine methyl ester hydrochloride (11) in 10 cc. of water were added 50 cc. of ether and the mixture cooled in an ice-salt bath. Anhydrous potassium carbonate was slowly added with shaking until the aqueous portion became a paste (12). The free ester was obtained by extraction of the paste with ether, and the ethereal solution diluted with an equal volume of ethyl acetate and dried over anhydrous potassium carbonate at 0°.

O-Acetyl-N-carbobenzoxy-l-tyrosyl-ε-carbobenzoxy-l-lysine Methyl Ester (III)—3.5 gm. of O-acetyl-N-carbobenzoxy-*l*-tyrosyl chloride in dry ether suspension were added in two portions with shaking to the above ether-ethyl acetate solution of the free carbobenzoxy-*l*-lysine methyl ester. After addition of the first portion, ε-carbobenzoxy-*l*-lysine methyl ester hydrochloride crystallized from the solution. The mixture was allowed to stand 5 minutes and the second portion of acid chloride added together with an aqueous solution of 1.7 gm. of potassium carbonate. The mixture was shaken for 15 minutes, transferred to a separatory funnel, and a few cc. of pyridine added to decompose the excess acid chloride. 200 cc. of water and 200 cc. of ether were added and the mixture shaken vigorously. O-Acetyl-

N-carbobenzoxy-*l*-tyrosyl-ε-carbobenzoxy-*l*-lysine methyl ester separated as a flocculent precipitate between the two layers. It was collected by centrifugation and washed with dilute hydrochloric acid, bicarbonate solution, and water. Yield, about 4.0 gm. On recrystallization from methyl alcohol, the substance melted at 138° (uncorrected).

$C_{34}H_{39}O_9N_3$.   Calculated.   C 64.45, H 6.16, N 6.63, —COCH₃ 6.80
633   Found.   " 64.63, " 6.17, " 6.90,   "   8.06
                          " 64.40, " 6.19, " 6.76

In several runs, a product was obtained which after recrystallization from methyl alcohol melted at 156°. In view of certain considerations discussed in the theoretical part of this paper, this compound appears to be the deacetylated ester.

$C_{32}H_{36}O_8N_3$.   Calculated.   C 65.02, H 6.12, N 7.12, —COCH₃ 0.00
590   Found.   " 64.62, " 6.24, " 6.32,   "   2.84
                          " 64.52, " 6.08, " 6.41

N-Carbobenzoxy-l-tyrosyl-ε-carbobenzoxy-l-lysyl Hydrazide (*IV*)—2.0 gm. of the acetyl dicarbobenzoxytyrosyl-*l*-lysyl methyl ester dissolved in warm methyl alcohol were treated with 0.6 cc. of hydrazine hydrate. On standing at room temperature overnight, 1.5 gm. of the crystalline hydrazide separated. For analysis, the substance was purified by solution in hot methyl alcohol and concentrated *in vacuo* until crystallization occurred; m.p. 210°.

$C_{31}H_{36}O_7N_5$.   Calculated.   C 62.94, H 6.10, N 11.85
590   Found.   " 62.47, " 6.09, " 11.48
                          " 62.59, " 6.16, " 11.20

The hydrazide prepared from the higher melting ester (m.p. 156°), presumably the deacetylated compound, also melted at 210°. No depression in melting point was noted with a mixture of the two hydrazides.

N-Carbobenzoxy-l-tyrosyl-ε-carbobenzoxy-l-lysyl-l-glutamyl-l-tyrosine Diethyl Ester (*VIII*)—1.5 gm. of N-carbobenzoxy-*l*-tyrosyl-ε-carbobenzoxy-*l*-lysyl hydrazide were ground in a mortar with 20 cc. portions of warm 75 per cent acetic acid. 225 cc. of this acid were required to produce a clear solution. After filtering, 113 cc. of water were carefully added to bring the acetic acid concentration to 50 per cent, and yet maintain complete solution. It was cooled to 4° and treated with an aqueous solution of 210 mg. of sodium nitrite. The azide crystallized out on standing in the ice bath for 10 minutes. The reaction mixture was diluted with 300 cc. of ice-cold water and extracted with cold ethyl acetate. The ethyl acetate solution was washed with ice water, cold bicarbonate solution, and again

with water. After drying over anhydrous magnesium sulfate at 0°, the solution was filtered directly into an ethyl acetate solution of *l*-glutamyl-*l*-tyrosine diethyl ester prepared from 1.6 gm. of the monoester. On standing overnight a small amount of a gelatinous precipitate formed. This was removed by filtration and the clear ethyl acetate solution thoroughly washed with water, dilute hydrochloric acid, aqueous bicarbonate, and again with water. On concentration of the dried ethyl acetate solution, crystallization occurred. Yield about 1.0 gm. The substance was recrystallized from alcohol-ethyl acetate mixtures and 95 per cent ethyl alcohol; m.p. 204°.

$C_{49}H_{58}O_{13}N_5$. Calculated. C 63.57, H 6.38, N 7.57, OC_2H_5 9.72, NH_2-N 0.00
                 Found. " 63.21, " 6.44, " 7.57, "   9.90, "   0.01
                        " 63.15, " 6.46, " 7.87,   "   0.00

Mol. wt., calculated, 925
7.910 mg.; 28.75 mg. camphor; depression 10.4°, 10.6°; mol. wt. 979, 960
2.305 "   31.57 "   "   · "   2.9°, 2.7°; "   " 931, 1000

l-Tyrosyl-l-lysyl-l-glutamyl-l-tyrosine (*IX*)—230 mg. of the dicarbobenzoxy tetrapeptide ester were dissolved in 15 cc. of 1 N NaOH by shaking at room temperature for 1 hour. A small amount of insoluble material was removed by filtration and the cooled filtrate acidified to Congo red with dilute hydrochloric acid. The product was collected by centrifugation, washed with water, dissolved in dilute sodium carbonate solution, and reprecipitated with hydrochloric acid. The wet material was washed with water, dissolved in 25 cc. of alcohol, and hydrogenated in the presence of palladium black and a drop of glacial acetic acid. A few drops of water were added occasionally to keep the product in solution. When hydrogenation was complete, the catalyst was filtered off and the solution concentrated *in vacuo*. A crystalline product was obtained by repeated addition and removal of absolute methyl alcohol. The residue was recrystallized from an ethyl alcohol-water mixture and dried at 60° over phosphorus pentoxide for 1 hour. On concentration of all mother liquors and recrystallization from the same solvent mixture, a total yield of 160 mg. was obtained.

$C_{29}H_{39}O_9N_5 \cdot 4H_2O$. Calculated. C 51.70, H 6.95, N 10.55, NH_2-N 4.16
673   Found.   " 51.62, " 6.60, " 10.09,   "   4.20

The 4 molecules of water of crystallization could not be determined in the usual manner because of the instability of the peptide at temperatures high enough to remove the water quantitatively. The evidence for the presence of 4 molecules of water rests in the ratio of total N to NH_2-N as well as in the results of the enzymatic studies described below. ·

Enzymatic Studies

Enzymes—Carboxypeptidase was prepared according to Anson (13) and recrystallized four times. Crystalline pepsin, trypsin, and chymotrypsin were prepared according to the directions of Northrop (14). All enzyme preparations had previously been tested for their activity, typical synthetic substrates and angiotonin being used (3, 6).

Incubation—A standard solution of the tetrapeptide was prepared by dissolving 139 mg. in 25 cc. of water. For each of the experiments recorded in Figs. 2 to 4, 5.00 cc. of the standard solution were diluted to 10.00 cc. with the desired buffer containing the requisite amount of enzyme. The final substrate concentration was therefore 0.1 mM, which corresponds to 0.0585 mg. of amino nitrogen per cc. of test solution for each amino group. Since there are two free amino groups in the original substrate, the blank value in each experiment was 0.117 mg. of NH_2-N per cc.

0.1 M citrate or phosphate buffers were used to maintain the acidity at the desired value.

The course of the hydrolysis was followed by the nitrous acid method of Van Slyke and the amino acid carboxyl in the chymotrypsin experiment was determined by the ninhydrin method of Van Slyke, Dillon, MacFayden, and Hamilton (9).

SUMMARY

The tetrapeptide, tyrosyllysylglutamyltyrosine, has been synthesized by a method which establishes its structure. It was accomplished by condensation of the two dipeptides, tyrosyllysine and glutamyltyrosine, whose functional groups were protected. These protecting groups were removed, freeing the desired optically active tetrapeptide in good yield. This compound did not affect the arterial blood pressure of a pithed cat in doses of 20 mg. per kilo of body weight.

The tetrapeptide was subjected to enzymatic hydrolysis by crystalline carboxypeptidase, chymotrypsin, trypsin, and pepsin. It was hydrolyzed by these four enzymes at the expected rate, thus supporting the specificity requirements for proteolytic enzymes as suggested by Bergmann and his associates.

The rate of hydrolysis under the influence of carboxypeptidase and pepsin was similar to the rate of inactivation of angiotonin by the same enzymes. Chymotrypsin and trypsin hydrolyzed the tetrapeptide very much more slowly than angiotonin. It was suggested that the sensitivity of angiotonin to tryptic hydrolysis is due to an as yet unknown amino acid arrangement.

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ON THE MECHANISM OF INVASION

I. ANTINVASIN I, AN ENZYME IN PLASMA

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The presence of a mucopolysaccharide, hyaluronic acid, in skin (1) and of a specific enzyme, hyaluronidase, in pathogenic bacteria (2–5), venoms (6), and spermatozoa (7–9) suggests that this enzyme plays an important rôle in processes of invasion by depolymerization of the mucoid ground substance of connective tissue. Additional evidence for the rôle of the enzyme in increasing the permeability of the host tissue has been contributed by the observation that hyaluronidase and "spreading factor" are identical (3, 10–12). According to Duran-Reynals (13), the degree of invasiveness of bacteria is largely determined by the amount of spreading factor present, and the successive phases of infection induced by invasive bacteria include (1) the hydrolysis of the mucoid ground substance of the connective tissue (a preexisting physiological obstacle); (2) spreading primarily through the interstitial system of the connective tissue.

Agreement seems to exist concerning the rôle of hyaluronidase as an invasion-promoting tool, but the defense mechanism of the body against the action of hyaluronidase has remained obscure.

In this and in Papers II and III three new enzymes will be reported. Two of these are part of the defense mechanism of the body, while the third promotes the invasion of bacteria and venoms. There are indications of two additional enzymes acting in this scheme, one observed in the plasma, the other in the invading organism.

A synonymous term for hyaluronidase, on account of its property of promoting spreading and invasion, could well be invasin. For this reason the terms "antinvasin" and "proinvasin" have been coined for the enzymes to be described.

The first enzyme to be described in this paper has been found in normal blood plasma of mammals, birds, and fish. This enzyme rapidly destroys hyaluronidase and, due to this property, acts as an antinvasive catalyst. It will be referred to as antinvasin I.

The second enzyme, proinvasin I, found in bacteria and venoms, will be described in Paper II. It rapidly inactivates antinvasin I, thus paralyzing the body defense mechanism, and indirectly promotes invasion.

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The third enzyme, antinvasin II, has been observed in normal plasma. It acts by destroying proinvasin I, thereby indirectly counteracting invasion. Tests for the quantitative determination of these three enzymes have been devised. The existence of a fourth enzyme, proinvasin II, which inactivates antinvasin II, and a fifth enzyme, antinvasin III, which destroys proinvasin II, can be predicted from observations made on *Staphylococcus aureus*. A quantitative test for these enzymes has, as yet, not been established. From the observations *in vitro* it would seem that certain problems of invasion and immunity may be understood and explained by the action of distinct catalysts occurring in plasma, spermatozoa, bacteria, and venoms.
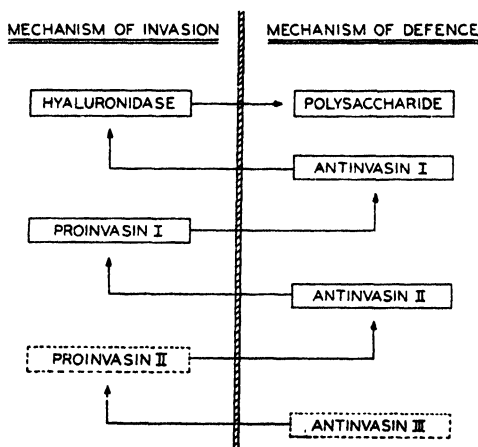
FIG. 1. Scheme of invasion

Fig. 1 shows in a schematic arrangement these various enzymes in the sequence in which, according to our evidence, they react with each other and with the polysaccharide.

Antinvasin I—There exists in the literature a number of somewhat contradictory observations reporting the presence in serum of an inhibitor of hyaluronidase. Thus, for example, by use of the decapsulation of virulent bacteria as a test for hyaluronidase activity (14), it was found that heterologous as well as homologous antisera inhibited the action of the enzyme. The presence of a substance in horse serum inhibiting the action of the "diffusion factor" has been mentioned briefly (11). However, with the depolymerization of hyaluronic acid (10), the hydrolysis of hyaluronic acid (15), the formation of a mucin clot (16), or the velocity of spreading in the skin (13) as the methods of assay, it was concluded that (1) the effect of each antiserum is limited to the preparation used for immunization, (2) inhibition

of hyaluronidase activity by antiserum is strictly specific, and (3) heterologous and normal sera exert no effect.

In contrast to the above observations, it will be demonstrated here that normal plasma contains a non-specific, highly active enzyme, which will be referred to as antinvasin I. This enzyme destroys the enzymatic activity of hyaluronidases derived from various sources. The mechanism of action of this enzyme as well as its distribution in various animals has been investigated. The reaction of antinvasin I with hyaluronidases obtained from testes, bacteria, and venoms has been studied; the properties of the enzyme and a method of assay are described.

Preparation of Test Substances

Polysaccharide—Hyaluronic acid, an acid polysaccharide, was first isolated by Meyer and Palmer from a variety of human and animal sources (17). Their procedure for extraction of the polysaccharide from umbilical cord has been applied here, with some modifications, because it was observed that yield and quality of the isolated polysaccharide depend considerably on the experimental conditions.

Human umbilical cord, washed free of blood, is stored in the ice box under acetone. The polysaccharide under these conditions remains unchanged for several months. For the extraction of the polysaccharide, the cord is minced and the acetone is pressed out; 120 gm. of cord, mixed with 70 gm. of sand, 250 cc. of water, and 100 cc. of Hayem's solution, are kept for 7 days at 2° and ground once daily for 15 minutes. The viscous solution which results is separated by centrifugation; the residue is twice reextracted by incubation with 200 cc. of water for a day. To the combined solutions acetate buffer of pH 4.7 is added to make a final concentration of 0.04 M, and the polysaccharide is separated by pouring the chilled solution into the 4-fold volume of cold acetone. After the precipitate is washed with acetone and dried *in vacuo* over $CaCl_2$, the polysaccharide is obtained in stable form as a white powder; yield, 3.4 gm. No denaturation or depolymerization takes place in the process of separation; viscosity and enzymatic activity are fully recovered by solution of the dry powder in 0.02 M acetate buffer of pH 4.7 and storage at low temperature.

Hyaluronidase from Testes—Claude and Duran-Reynals (18) have described a method for the preparation of spreading factor from testicular extracts. A somewhat modified procedure was used here for the isolation and purification of hyaluronidase. Testes of horse and hogs, trimmed of connective tissue, were minced in a meat grinder, extracted with acetone, and dried in a stream of air. A very active enzyme was obtained by extraction of the dry powder with 0.1 N acetic acid, fractionation with

ammonium sulfate between 0.3 and 0.7 saturation, and dialysis of the
neutralized solution.   Hyaluronidase from bovine testes, purified according
to the procedure of Madinaveitia (19), was obtained through the kindness
of Dr. Erwin Schwenk of the Schering Corporation.

Hyaluronidase from Pneumococcus Type II and Type VI—The method
for the preparation of hyaluronidase from pneumococcus was essentially
the same as that described by Meyer *et al.* (4) in the isolation of the enzyme
from *Clostridium welchii*.   Bacto-brain-heart infusion broth (Difco Labora-
tories) was inoculated with cultures of pneumococcus type II and type VI
(from the Antitoxin and Vaccine Laboratory, Boston) and incubated for
17 hours at 37.5°.   The cells were collected by centrifugation and sus-
pended in a small amount of water with an excess of toluene.   The enzyme
was brought into solution by autolysis at 37.5° for 2 days and cell fragments
were removed by centrifugation.   Hyaluronidase was finally obtained in
purified form by fractionation at pH 4.7 with ammonium sulfate between
0.3 and 0.8 saturation and subsequent dialysis.

The course of events in the reaction of bacterial enzymes with plasma
enzymes is determined essentially by the concentration of these biocatalysts.
To simulate physiological conditions as much as possible it seemed
especially important not to alter the relative proportion between the
various bacterial enzymes in the process of isolation.   Accordingly, the
enzymes employed in this study were obtained by concentration of the
culture medium into which the enzymes had been released spontaneously
by the growing cells.   No fractionation was used other than dialysis, which
removed salts and metabolites.

Hyaluronidase and Proinvasin I from Pneumococcus Type VI—The
enzymes were obtained from the supernatant solution of a 48 hour culture
of pneumococcus after separation of the cells, concentration, and dialysis.
Hyaluronidase prepared from the medium is about 70 times less pure than
that isolated by autolysis of the cells and subsequent fractionation.   In
their reaction with antinvasin I, however, both products are alike and the
method of preparation of enzymes from the culture medium has been
adopted for other pathogens.

Hyaluronidase and Proinvasin from Staphylococcus aureus—For the
cultivation of *Staphylococcus aureus*, brain-heart infusion broth was heavily
inoculated and incubated at 37.5° for 8, 24, and 48 hours respectively.
Hyaluronidase (3, 4), as well as the proinvasin, appears in the culture me-
dium during growth of the bacteria and they remain in the solution when the
organisms are removed by centrifugation.   In this respect the enzymes
behave like exotoxins.   The absolute amount of the enzymes released into
the medium increases considerably with the time of incubation, the con-
centration of hyaluronidase increasing faster than that of proinvasin.   A

concentrated solution of the enzyme was prepared from the 24 hour culture. The supernatant fluid, after removal of the bacteria, was put into a cellophane bag and concentrated by evaporation in a strong current of air over a hot-plate. Precaution was taken to keep the temperature inside the bag below 35°. The concentrated enzyme solution finally was dialyzed to remove phosphate and other interfering substances.

Hyaluronidase and Proinvasin from Clostridium welchii, Type B—A stock culture of this organism was furnished by the American Type Culture Collection of Georgetown University, Washington, D. C. In the preparation of the enzymes from *Clostridium welchii* the same procedure was followed as that described for *Staphylococcus aureus*, except that the organisms were cultivated in thioglycollate medium (No. 135 of the Baltimore Biological Laboratory). The organisms grew exceptionally well in this medium.

Hyaluronidase and Proinvasin I from Snake Venom—Venom from two different types of poisonous snakes was used in this investigation. Both enzymes are present in these venoms in large amounts, although their relative concentration varies with the species. The venom obtained from the diamondback rattlesnake (*Crotalus adamanteus*) proved to be a richer source of hyaluronidase than that of the moccasin (*Agkistrodon piscivorus*). The concentration of proinvasin I was found to be 10 times higher in the moccasin than in the rattlesnake. The purified and concentrated venoms were supplied by Ross Allen, Silver Springs, Florida, and they were kept as dry preparations at room temperature for several months.

Test

A method of measuring quantitatively the fall in viscosity due to the depolymerization of hyaluronic acid was used for the assay of hyaluronidase, of antinvasin, and of proinvasin. Under defined conditions of viscometric assay similar to those used by Chain and Duthie (3), and Madinaveitia and Quibell (20), hyaluronidase will decrease the viscosity of the polysaccharide at a rate directly proportional to its concentration. The test for hyaluronidase was set up in such a way that it could be used also for the quantitative determination of the other components in the chain of enzymes. Conditions have been so arranged that any one of the enzymes indicated in Fig. 1 can be made the rate-determining factor. The rate of breakdown of the polysaccharide will then be proportional to hyaluronidase and to proinvasin or it will be inversely proportional to antinvasins I and II. The test thus represents a model for the situation prevailing in the body with its continuous interplay between aggression and defense. It has been stated only very recently (21) that "the viscosimetric method is beset with difficulties." The present investigation depends mainly on the exact

measurement of viscosity and a somewhat detailed description of the experimental conditions may be in order.

Test for Hyaluronidase—The enzyme, dissolved in borate buffer, and the polysaccharide, buffered by phosphate, were incubated separately at 25°. The rapid depolymerization of the polysaccharide, taking place after com-

TABLE I

Test for Hyaluronidase

Ostwald viscosimeter, capillary diameter, 0.48 mm.;* hyaluronidase from bovine testicle; polysaccharide, 6 mg. per cc. of 0.02 M acetate buffer, pH 4.7;† temperature, 25°.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
0.2 M borate buffer, pH 6.7, cc.	0.25					
Water, cc.	0.60	→	→	→	→	→
Hyaluronidase, mg.			0.008	0.018	0.035	0.070

Solutions incubated 10 min., then combined

0.5 M phosphate buffer, pH 7.0, cc.	0.50	0.50				
2 M NaCl, cc.	0.25	0.25	→	→	→	→
Polysaccharide, cc.		1.00				
0.02 M acetate buffer, pH 4.7, cc.	1.00					
Flow time, sec.	$t_2 = 63.2$	$t_1 = 125.6$				
Half life time, R_0, sec.			1630	645	290	125
$\frac{1}{R_0} \times 10^3 \left[\frac{1}{\text{sec.}}\right]$			0.6	1.55	3.4	8.0

* The viscosimeter substituted later had a capillary of 0.56 mm. in diameter. The time required for measurement of the viscosity thereby was reduced to one-half. Identical values for the relative viscosity were found, independent of these variations in the dimension of the capillary.

† The polysaccharide concentration was increased later to 8 mg. per cc. to increase the accuracy of the test.

bination of the two components, is measured at frequent intervals by following the decrease in viscosity.   Experimental details are given in Table I and Fig. 2, while Table II illustrates the method of calculation.

The decrease of viscosity as a function of hyaluronidase concentration and time is shown in Fig. 2.

The time required to diminish the viscosity of the polysaccharide to

TABLE II
Calculation of Relative Viscosity and Reaction Time

t = time after addition of hyaluronidase; t_1 = flow time of the solution containing buffer, salt, enzyme, and polysaccharide; t_2 = flow time of the solution containing buffer, salt, and enzyme; t_3 = reaction time = $[t + 0.5t_1]$; η = relative viscosity = $[(t_1/t_2) - 1]$; R_0 = reaction time required for 50 per cent diminution of viscosity (half life time).

Experiment 5, Table I; concentration of hyaluronidase, 0.035 mg.

t	t_1	t_2	t_3	η
sec.	*sec.*	*sec.*	*sec.*	
0	125.6	63.2		0.99
47	107.4	63.2	100	0.70
183	97.4	63.2	232 R_0	0.54
307	92.8	63.2	353	0.46
433	89.2	63.2	478	0.41
600	86.2	63.2	643	0.36

Half life time, R_0 = 290 seconds.
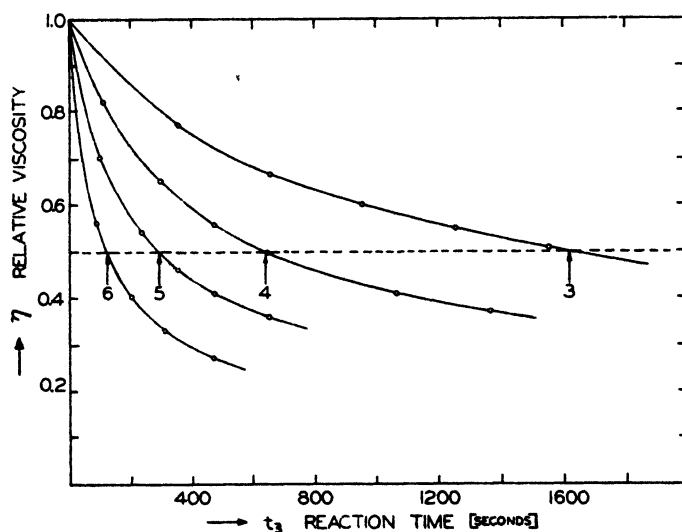
FIG. 2. Depolymerization of the polysaccharide as a function of bovine testis hyaluronidase. Experiment 3, 0.008 mg. of hyaluronidase; Experiment 4, 0.018 mg.; Experiment 5, 0.035 mg.; Experiment 6, 0.070 mg.

50 per cent of the original level (half life time = R_0) was determined by interpolation of the graphs in Fig. 2. This time is approximately inversely proportional to the enzyme concentration, and by plotting $1/R_0$ as the function of the hyaluronidase nearly straight lines were obtained (Fig. 3).

The enzymatic activity of hyaluronidase from all these sources apparently can be determined with a high degree of accuracy. No inactivation of the enzyme takes place and the rate of reaction remains constant for the duration of the experiment.

Test for Antinvasin I—Antinvasin I, the enzyme in blood plasma, reacts by destroying hyaluronidase. Activity and concentration of antinvasin I can be determined quantitatively by measuring the amount of hyaluronidase that has been destroyed in a given time. The rate of this reaction depends upon the concentration of the two enzymes, the reaction time, the temperature, the hydrogen ion concentration, and other factors like
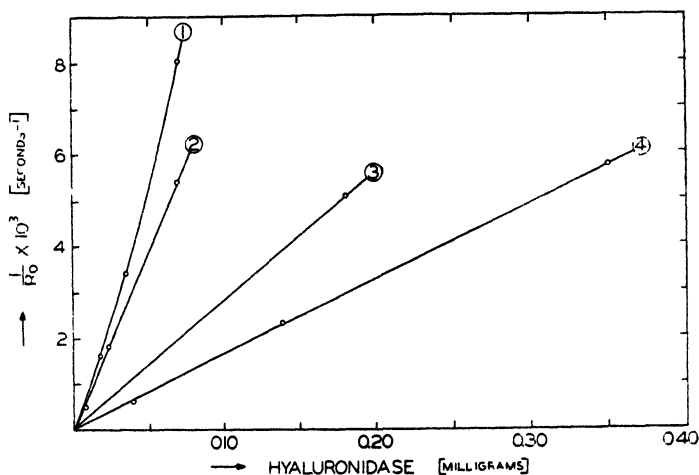
FIG. 3. Test for hyaluronidase. Curve 1, hyaluronidase from bovine testes; Curve 2, from type VI pneumococcus; Curve 3, from rattlesnake venom; Curve 4, from moccasin venom.

salt and protein concentration. Phosphate will completely inhibit the reaction of antinvasin I with hyaluronidase, while it does not interfere in the reaction of hyaluronidase with the polysaccharide. Experimental conditions of the test and mathematical treatment of the results can be simplified considerably with this arrangement because a clear cut separation of the two enzymatic processes becomes feasible. In the test described in Table III hyaluronidase is first incubated with antinvasin I of chicken plasma. Phosphate is absent during this period while the reaction between antinvasin I and hyaluronidase is in progress. The fraction of hyaluronidase remaining intact after its exposure to antinvasin I is then measured in the viscosimeter (Fig. 4). During this second part of the experiment sufficient phosphate is present to prevent further activity of antinvasin I.

Plasma contains no hyaluronidase, since no depolymerization takes place

TABLE III

Test for Antinvasin I

Ostwald viscosimeter, capillary 0 48 mm; hyaluronidase from bovine testicle; polysaccharide, 6 mg. per cc. of 0.02 M acetate buffer, pH 4.7.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6	Experiment 7
0 2 M borate buffer, pH 6 7, cc	0 25	0 25	0 25	0 25	0 25	0.25	0 25
Water, cc	0 60	0.60	0 60	0 40	0.40	0.50	0.40
Plasma of chicken (antinvasin I), cc				0.20	0.20	0.10	0.20
Hyaluronidase, mg			0 05			0.05	0.05
Solutions incubated 10 min at 25°, then combined							
0.5 M phosphate buffer, pH 7 0, cc	0 50	→	→	→	→	→	→
2 M NaCl, cc	0 25	→	→	→	→	→	→
Polysaccharide, cc	1.00	1.00	1 00	1 00	1 00	1.00	1.00
0.02 M acetate buffer, pH 4 7, cc							
Flow time, sec.	$t_2 = 63\ 2$	$t_1 = 128\ 5$		$t_2 = 65\ 3$	$t_1 = 131.8$		
Half life time, R_0, sec.			170			640	990
" " " R, "							
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$						2 8	4.8
Destruction of hyaluronidase in 10 min at 25° $\left[D = 100\ \dfrac{A}{A+1} \right]$, %						74	83

after its addition to the polysaccharide. The viscosity of the test solutions is slightly increased by plasma, which necessitates a small correction.

The method of calculating enzymatic activity from viscometric data was described in Table II. It has been extended here and applied for the determination of antinvasin I. The decrease in viscosity as a function of time is plotted in Fig. 4 for various concentrations of antinvasin I.
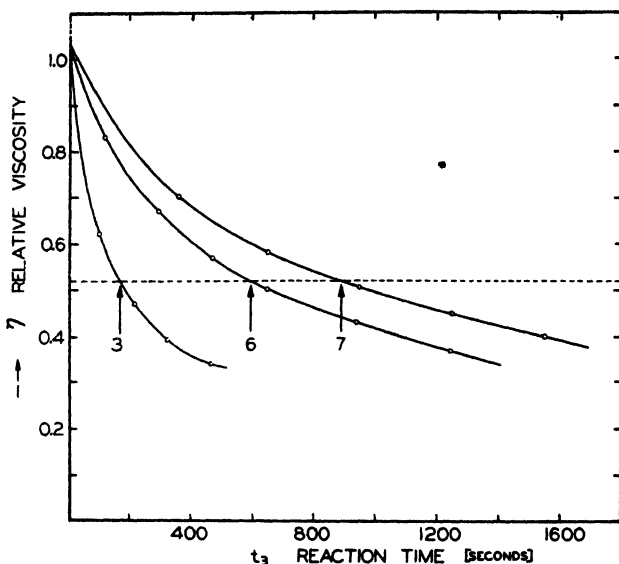
FIG. 4. Depolymerization of the polysaccharide as a function of antinvasin I of chicken plasma. Experiment 3, without plasma; Experiment 6, with 0.10 cc. of plasma; Experiment 7, with 0.20 cc. of plasma.

From the graphs in Fig. 4 the half life time of the polysaccharide, under various conditions, can be derived. If we designate

R_0 = half life time before reaction of hyaluronidase with plasma
R = " " " after " " " " "
A = activity of antinvasin I
D = % of destruction of hyaluronidase in 10 min. at 25° by antinvasin I

then

$$(1) \quad A \quad \frac{R - R_0}{R_0} \qquad\qquad (2) \quad D = \frac{}{A + 1} \times 100(\%)$$

The activity of antinvasin I (A) was calculated from the data obtained in Table III and Fig. 4 and from similar experiments performed with human, hog, and chicken plasma. The extent to which the equation (1) conforms

with the experimental results has been determined in Fig. 5 by plotting activity of antinvasin I as a function of the antinvasin I concentration of plasma.

The results illustrated in Fig. 5 indicate that under the conditions described an accurate determination of antinvasin I in various sources appears feasible. The test furthermore demonstrates the high activity of antinvasin I. By means of equations (1) and (2), it can be calculated that the enzyme present in 0.05 cc. of human plasma is capable of destroying as much as 83 per cent of hyaluronidase in 10 minutes under the conditions of Table III.
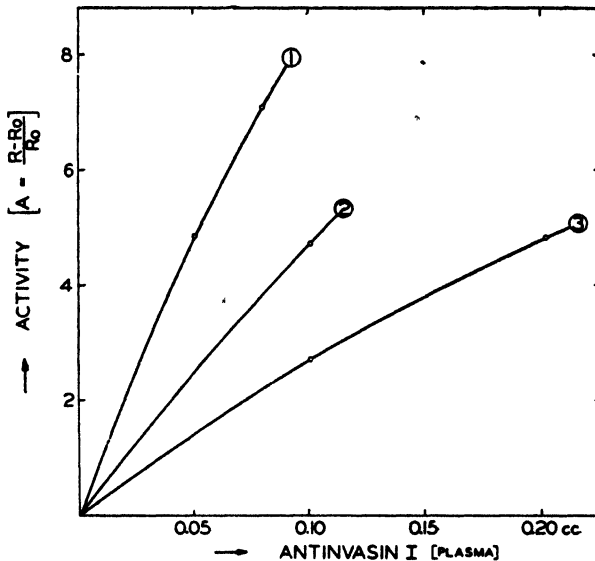
FIG. 5. Test for antinvasin I in blood plasma of (Curve 1) human, (Curve 2) hog, and (Curve 3) chicken. (Hyaluronidase of bovine testicle.)

It is conceivable that antinvasin I also is responsible for the destruction and elimination of spreading factor from plasma; Duran-Reynals (22) 12 years ago observed rapid disappearance of spreading factor that had been introduced into the blood stream by intratesticular infection with staphylococci.

Enzyme Kinetics—The test described in Table III and Fig. 5 can be employed to study the mechanism of action of antinvasin I, its properties, and its distribution in nature. It seemed of particular interest to investigate the kinetics of the enzyme reaction in order to arrive at a better understanding of the delicate equilibrium conditions which seem to determine the course of interaction of the different components of this system. It will be

TABLE IV

Activity of Antinvasin I As Function of Time

Experimental conditions as in Table III.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
0.2 M borate buffer, pH 6.7, cc.........	0.25	0.25		
Water, cc...............................	0.60	0.50	$\rightarrow$	$\rightarrow$
Plasma (chicken), cc...................		0.10		
Hyaluronidase, mg.....................	0.05	0.05		

Incubated at 25°				
		5 min.	10 min.	20 min.
0.5 M phosphate buffer, pH 7.0, cc......	0.50			
2 M NaCl, cc...........................	0.25	$\rightarrow$	$\rightarrow$	$\rightarrow$
Polysaccharide, cc.....................	1.00			
Half life time R_0, sec.	170			
" " " R, sec...		510	640	810
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$		2.0	2.8	3.8
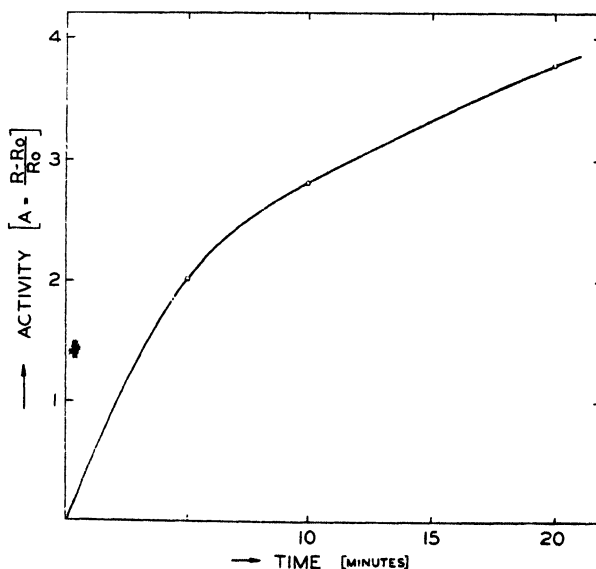
FIG. 6. Activity of antinvasin I as a function of time

possible to arrive at a clear understanding of these vital processes only if the reaction rates as well as the concentrations of the individual catalysts are considered in both systems; *i.e.*, in the invading organism and in the

defense mechanism of the host. On the basis of the physicochemical data it will be possible also to distinguish between the enzymatic nature of the catalysts considered here and antibody reactions postulated previously.

Effect of Time on Reaction, Antinvasin I-Hyaluronidase—The time factor in this reaction has been investigated by incubation of constant amounts of hyaluronidase and antinvasin I (chicken plasma) for various periods of time. The conditions of these experiments are given in Table IV and the results are shown in Fig. 6.

Fig. 6 illustrates that the reaction of antinvasin I with hyaluronidase depends considerably on the time of reaction; after 20 minutes about 80 per cent of hyaluronidase was destroyed. The speed of antibody reaction is in marked contrast to the comparatively slow rate of this enzyme reaction. Mayer and Heidelberger (23) have obtained evidence that combination between pneumococcus polysaccharides and their antibodies is at least 90 per cent complete in less than 3 seconds.

Effect of Concentration on Reaction, Antinvasin I-Hyaluronidase—The method described in Table III has been employed for the study of the rate of destruction of hyaluronidase by antinvasin I in which k represents the specific velocity constant of the following reaction:

$$- \frac{\Delta \text{ Hyaluronidase}}{\Delta t} = k \times (\text{hyaluronidase}) \times (\text{antinvasin I})$$

We designate

R_0 = half life time before reaction with antinvasin I
R = "   "   " after   "   "   "   "
C_0 = concentration of hyaluronidase before reaction with antinvasin I
C = "   "   " after   "   "   "   "
$\frac{\Delta C}{\Delta t}$ = destruction of hyaluronidase in 10 min. at 25° $\left[\frac{- \Delta \text{ hyaluronidase}}{\Delta t} \right]$

According to Fig. 3,

$$C \times R = \text{constant or (3) } C \times R = C_0 \times R_0$$

$$(4) \qquad C = C_0 \times \frac{R_0}{R}$$

$$(5) \qquad \frac{\Delta C}{\Delta t} = C_0 - C$$

$$(6) \qquad \frac{\Delta C}{\Delta t} = C_0 \left[1 - \frac{R_0}{R} \right]$$

The rate of reaction as a function of hyaluronidase has been studied in Table V. Increasing amounts of hyaluronidase were added to a constant amount of antinvasin I and the rate of destruction of hyaluronidase was measured after incubation for 10 minutes at 25°.

The results of this experiment are given in Fig. 7. The rate of destruction of hyaluronidase by antinvasin I is directly proportional to the concentration of hyaluronidase. This observation reveals a fact of considerable

TABLE V

Rate of Reaction As Function of Hyaluronidase Concentration

Experimental arrangement and test substances as in Table III; source of antinvasin I, 0.10 cc. of plasma of chicken.

Experiment No.	Hyaluronidase	Half life time		Rate of reaction
	C_0	R_0	R	$\frac{\Delta C}{\Delta t} = C_0 \left[1 - \frac{R_0}{R} \right]$
	mg.	*sec.*	*sec.*	*mg. per 10 min.*
1	0.03	290	1180	0.022
2	0.06	175	500	0.039
3	0.09	105	265	0.055
4	0.12	58	143	0.072
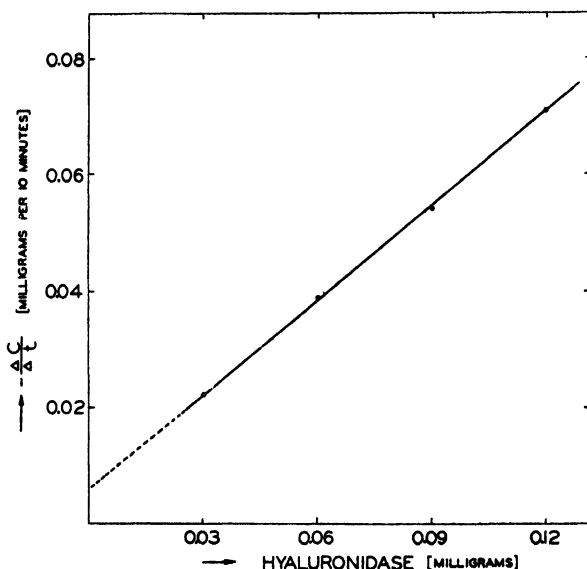
FIG. 7. Activity of antinvasin I as a function of hyaluronidase concentration

physiological significance, since it demonstrates that antinvasin I, present in normal plasma, has the capacity of destroying large amounts of hyaluronidase. Activity and concentration of the defense enzyme are apparently of such a magnitude that it can deal effectively with high concentrations of hyaluronidase, corresponding to a great number of invading organisms.

From an inspection of Fig. 7 it becomes apparent that the activity of antinvasin I varies greatly with the concentration of hyaluronidase. In the analytical test for antinvasin I it is therefore necessary to work with a constant amount and a standard preparation of hyaluronidase.

<div align="center">

TABLE VI

Rate of Reaction As Function of Antinvasin I Concentration

</div>

Experimental conditions and test substances as in Table III; source of antinvasin I, plasma of chicken; hyaluronidase, 0.05 mg.

Experiment No.	Antinvasin I	Half life time		Rate of reaction
		R_0	R	$\dfrac{\Delta C}{\Delta t} = C_0 \left[1 - \dfrac{R_0}{R} \right]$
	cc.	*sec.*	*sec.*	*mg. per 10 min.*
1	0.05	170	370	0.027
2	0.10	170	640	0.037
3	0.20	170	990	0.042
4	0.30	170	1180	0.043
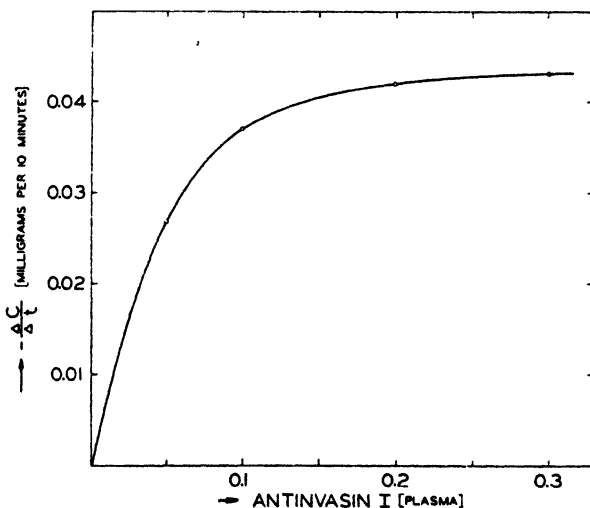
FIG. 8. Activity of antinvasin I as a function of antinvasin I concentration

The rate of destruction of hyaluronidase as a function of antinvasin I concentration has been investigated by combining small but constant amounts of hyaluronidase with various concentrations of antinvasin I. The data of this experiment are given in Table VI.

A plot of this experiment, shown in Fig. 8, demonstrates that proportionality between reaction velocity and concentration exists only at low

concentrations of antinvasin I. The reaction between antinvasin I and
hyaluronidase then is first order with respect to each of these constituents.
Formation of a dissociating antinvasin-hyaluronidase complex takes place
with increasing concentration of antinvasin I. It would be premature to
assign numerical values to the dissociation constant of this complex or to the
velocity constant of the reaction because so far only crude enzyme prepara-
tions have been employed.

Effect of Polysaccharide Concentration—In the course of preparation of
the polysaccharide it had been observed that the rate of reaction depended
considerably on the quality of the polysaccharide. The qualitative differ-
ence observed between different preparations of the polysaccharide made it
advisable to investigate also the effect of various concentrations of the poly-
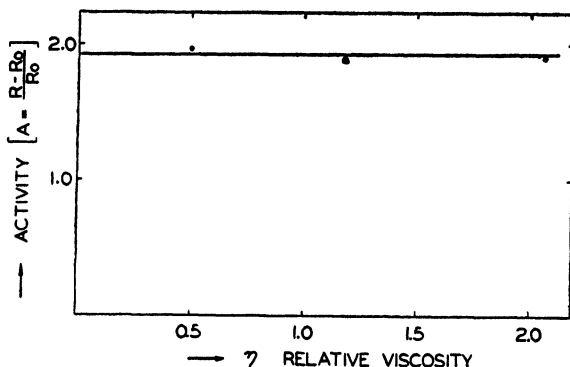
FIG. 9. Activity of antinvasin I at various polysaccharide concentrations

saccharide on the rate of reaction of antinvasin I. The activity of antin-
vasin I at various polysaccharide concentrations has been investigated in
the following experiment.

From the course of the reaction plotted in Fig. 9 it can be concluded
that antinvasin I activity can be determined independent of the poly-
saccharide concentration. Under the experimental conditions hyaluroni-
dase is saturated with its substrate. Furthermore, there seems to be no
competitive displacement of antinvasin I by an excess of polysaccharide.

pH Optimum—The pH activity curve for enzymatic destruction of
hyaluronidase by antinvasin I is shown in Fig. 10. Varying amounts of
HCl and NaOH were added to different aliquots of hog plasma and the
treated samples were allowed to react with bovine testicle hyaluronidase
at various pH values for 10 minutes at 25°. The antihyaluronidase activity
produced by each of the treated sera was measured in the test described in
Table III and was compared with that produced by a comparable amount of
untreated plasma.

Antinvasin I is much less active at pH 5.1 and pH 9.2 respectively than at neutral reaction. This is not due to an inactivation of the enzyme by hydrogen ions or hydroxyl ions, as in both instances full activity has been regained after readjustment to pH 6.7. The pronounced maximum of activity at pH 6.7 indicates that the velocity of the enzymatic reaction depends on the degree of ionization. The mechanism responsible for this optimum pH cannot be discussed at present. Only after the isoelectric points of both enzymes have been determined will it be possible to ascertain which represents the active form, the ion or the undissociated enzyme molecule.
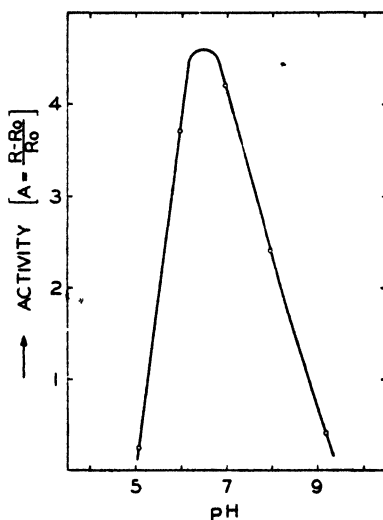
FIG. 10. Activity of antinvasin I as a function of pH

Effect of Temperature on Reaction, Antinvasin I-Hyaluronidase—The activity of antinvasin I, like that of other enzymes, increases with temperature. To test this relation quantitatively hyaluronidase was exposed to the action of antinvasin for 10 minutes at 0° and 25° respectively. The remaining hyaluronidase left intact after this time was measured at 25° and activity of antinvasin I calculated as described in Tables II and III.

The activity of the enzyme at various temperatures is shown in Fig. 11. A temperature increase from 0–25° results in a 6-fold increase in enzymatic activity, a value typical for an enzyme.

Inhibition by Phosphate, Effect of Salts—After observing the formation of an enzyme complex between antinvasin I and hyaluronidase, it seemed

of interest to consider the nature of the chemical bond reactive in this complex.   The participation of phosphate linkages in the combination of enzyme protein and prosthetic group has been established previously in respiratory enzymes (24–26).   Inorganic phosphate, in such cases, reacts as an inhibitor because it displaces the prosthetic group of the enzyme which is an organic phosphorus compound.   Antinvasin I, likewise, is completely and specifically inhibited by inorganic phosphate and for reasons of analogy it may be predicted that in this enzyme reaction organic phosphate bonds are also involved.
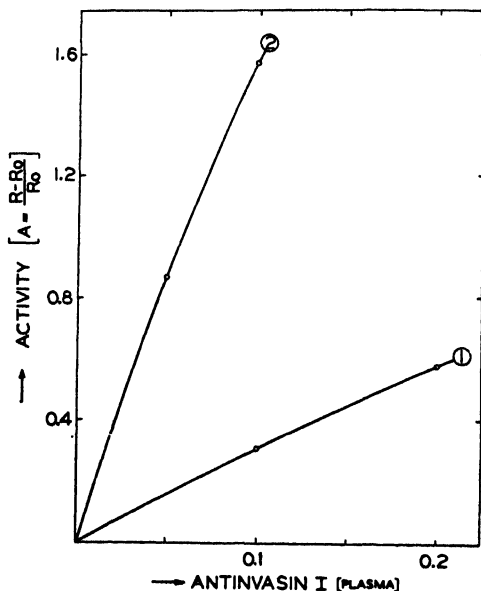
FIG. 11. Activity of antinvasin I as a function of temperature.   Curve 1, at 0°; Curve 2, at 25°.

Antinvasin I of hog plasma and hyaluronidase were incubated in phosphate buffer, pH 7.0, of various strengths, and the reaction was allowed to proceed at 25° for 10 minutes.   The enzymatic activity of antinvasin I was then determined under the conditions described in Table III.   The results of the experiment are shown in Fig. 12.

The effect of various electrolytes, sodium salts of weak and strong acids, has been investigated under the same conditions as described for phosphate by incubation of antinvasin I with hyaluronidase in neutral solution of the salts.   The results of this experiment arranged in the order of increasing ionization of the acids are compiled in Table VII.

The activity of antinvasin I is slightly increased by weak electrolytes,

while it is somewhat depressed by completely ionized electrolytes. The inhibition by phosphate is an exception to this rule. It is apparently caused by a specific reaction and not merely by a general salt effect. Cyanide,
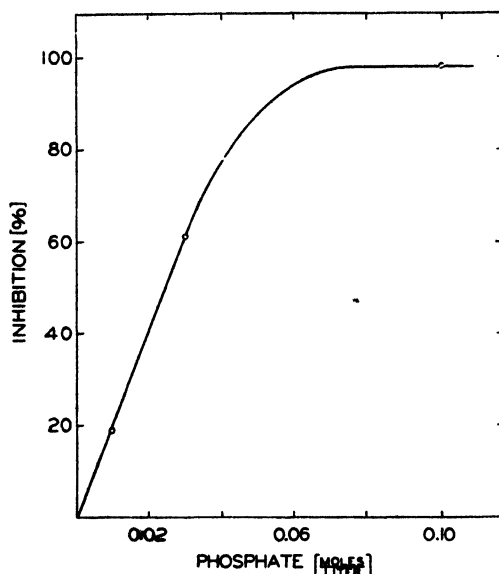
FIG. 12. Inhibition of antinvasin I by phosphate

TABLE VII

Effect of Salts on Antinvasin I

Salt, 0.1 M	Half life time		Activity of antinvasin I $\left[A = \frac{R - R_0}{R_0}\right]$	
	R_0	R		
	sec.	*sec.*		*per cent*
No added salt.................	195	820	3.2	
Borate........................	170	870	4.1	28 Acceleration
Cyanide.......................	170	820	3.8	19 "
Arsenate......................	180	870	3.8	19 "
Phosphate.....................	195	205	0.05	98 Inhibition
Chloride......................	195	680	2.5	22 "
Nitrate.......................	180	460	1.5	53 "
Sulfate.......................	170	420	1.5	53 "

even in high concentration, does not interfere with the activity of antin-vasin I, which seems to indicate that heavy metals like copper or iron are not involved in the reaction mechanism of this enzyme.

Stability of Antinvasin I—The resistance of the enzyme against denatur-

ing agents like heat, acid, and bases has been investigated, and the results are presented in Table VIII. A knowledge of these data is required not only in future attempts at purification and isolation but also for the identification of the enzyme and its distinction from other biologically active substances, like antibodies.

From the data accumulated in Table VIII certain conclusions can be drawn in regard to the chemical nature of antinvasin I. The fact that the enzyme does not dialyze through a cellophane membrane indicates a high molecular weight. The stability of the enzyme against prolonged dialysis shows that no splitting off of a prosthetic group occurs.

From the velocity of destruction at two temperatures the activation energy for enzyme inactivation [$\mu = \Delta H + RT$] has been calculated to correspond to 124,000 (calories per mole), which is a typical value for an

TABLE VIII

Stability of Antinvasin I (in Hog Plasma)

Treatment			Destruction
Time	Temperature	pH	
	°C		*per cent*
Storage, 16 days.................	2	7.5	0
Dialysis, 3 "	2	6.0	0
15 min............................	25	10.2	20
15 "	25	2.9	87
15 "	50	6.7	9
15 "	55	6.7	84

enzyme. The average value calculated for the destruction of thirty-four different enzymes was found to be $\mu = 68,600$ (27).

Substances like heparin (14), or antibodies (10, 13–16), have been reported to act as inhibitors of hyaluronidase, but antinvasin I can be distinguished from these substances by its response to denaturation. Antibodies are relatively thermostable (28), while the enzyme loses most of its activity in 15 minutes at 55°. While antinvasin I is destroyed by slightly elevated temperatures and in weakly acidic solutions, heparin is completely stable even under much more drastic conditions (29).

Source of Antinvasin I—Plasma containing antinvasin I was obtained from blood which had been defibrinated by stirring. 70 per cent less antinvasin I was found in serum after formation of the clot as compared with the yield obtained from defibrinated blood. This loss presumably is due to adsorption of the enzyme.

A few preliminary experiments were performed with various tissues in an attempt to obtain information about the origin of the enzyme, and with

the hope of finding a richer source than plasma as a material for future isolation of the enzyme. Various organs were minced and ground with sand, extracted with water for a few hours at room temperature, and then centrifuged. The supernatant solutions were tested for antinvasin I, but no activity was found in extracts from spleen, kidney, liver, heart, and stomach of hog or from the middle cervical lymph nodes of beef. None was found in laked red blood corpuscles. An appreciable amount was found in thymus but these extracts are very viscous and inconvenient to manipulate.

A number of observations has been reported in the preceding sections to demonstrate the enzymatic nature of antinvasin I and to distinguish it from antibodies. The enzymatic reaction described here may be taken as a mechanism of natural immunity against invading organisms, as distinguished from the acquired immunity represented by antibody formation following exposure to antigens. Antibodies appear in the circulation following exposure of the animal to an antigen; their concentration falls off rapidly after this contact ceases. In contrast, antinvasin is present in normal plasma of all species investigated. Furthermore, antibodies react specifically with the antigen used for immunization while antinvasin I, in a non-specific reaction, will destroy hyaluronidases from various sources. Experiments which exemplify this fundamental difference are described in the following section.

Distribution of Antinvasin I and Its Reaction with Hyaluronidase from Various Sources—The test described in Table III was used here to investigate the enzymatic reaction taking place after combination of antinvasin I in various plasmas with different hyaluronidases. The hyaluronidase concentration in every case was adjusted to correspond to a half life time of $R_0 = 175$ seconds.

It is concluded from the foregoing facts that the healthy organism possesses protective enzymes which, by destroying hyaluronidase, are destroying tools of invasion. These protective enzymes found in normal plasma of all species investigated appear to be an important factor in natural immunity. An inspection of the data compiled in Table IX reveals that in the majority of cases reaction between plasma antinvasin I and hyaluronidase has taken place. This point strongly indicates the non-specificity of the process and it seemed obvious to predict the presence of an interfering substance in those exceptional instances in which the plasma enzymes failed to react with hyaluronidase. In Paper II a second enzyme, proinvasin I, will be described which is responsible for this interference. Certain pathogenic bacteria and snake venoms not only contain hyaluronidase as a tool for invasion of the tissues but also produce additional enzymes like proinvasin to protect hyaluronidase against destruction by the body defense

TABLE IX

Reaction of Antinvasin I of Various Plasmas with Hyaluronidases from Different Sources

Source of hyaluronidase	Source of antin-vasin I; plasma	Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0}\right]$*	Destruction of hyaluronidase $\left[D = \dfrac{A}{A+1}100\right]$†	Average [D]†
			per cent	*per cent*
Horse testes	Human	4.9	83	
	Chicken	3.8	79	
	Rabbit	6.8	87	
	Beef	1.4	58	
	Horse	2.8	74	76
Bovine testes	Human	9.2	90	
	Hog	4.7	83	
	Chicken	4.5	82	
	Carp	2.1	68	
	Rabbit	1.0	50	
	Beef	0.32	24	66
Pneumococcus type VI	Human	9.3	90	
	Hog	0.85	46	
	Chicken	1.8	64	
	Rabbit	1.0	50	
	Beef	2.4	71	64
Pneumococcus type II	Human	4.1	80	
	Hog	0.66	40	
	Chicken	0.88	47	
	Rabbit	0.54	35	
	Beef	1.5	60	52
Clostridium welchii	Human	0.53	35	
	Hog	2.2	69	
	Chicken	0.1	9	
	Carp	0.02	2	
	Beef	10.2	91	41
Staphylococcus aureus	Human	0.53	35	
	Hog	0.17	15	
	Chicken	0	0	
	Rabbit	0	0	
	Beef	4.1	80	26
Snake venom, moccasin	Human	0	0	
	Hog	0	0	
	Chicken	0	0	
	Beef	0	0	
	Horse	0	0	0

* A, relative concentration of antinvasin I in 0.1 cc. of plasma.

† D, destruction of hyaluronidase in 10 minutes at 25°.

enzymes. The figures given in Table IX represent, therefore, minimum values for antinvasin I activity, depending upon the relative proportion of the enzymes proinvasin and hyaluronidase produced by the pathogenic organisms. Hyaluronidase from pneumococcus is accompanied by small amounts of proinvasin I and destruction of antinvasin I in this case proceeds at a slow rate. On the other hand, the larger amounts of proinvasin produced by *Staphylococcus aureus* or present in moccasin venom will destroy antinvasin I so rapidly that its rate of reaction with hyaluronidase becomes negligible. Proinvasin I rapidly destroys antinvasin I in plasma and it is conceivable that the *in vivo* action of proinvasin I may be the reason for the lowered concentration of antinvasin I observed in plasma of certain individuals.

At this point it will be necessary to consider the participation of still another enzyme present in plasma, antinvasin II. The data in Table IX demonstrate that the activity of antinvasin I of beef plasma is low with testicular hyaluronidase, although the same plasma showed exceptionally high activity with hyaluronidase isolated from *Staphylococcus aureus* and *Clostridium welchii*. This observation was the first indication of the existence of this new enzyme, which was found later to be present in every plasma investigated. A more detailed description of this enzyme will be presented in Paper III of this series. Antinvasin II reacts by destroying proinvasin I from various sources such as bacteria and snake venoms. At low concentration of antinvasin I, for example that prevailing in beef plasma or in the plasma of certain patients, reaction (b) proceeds so slowly that it becomes negligible compared to reaction (c).

(a)	Hyaluronidase	+ antinvasin I	→	inactivated hyaluronidase
(b)	Antinvasin I	+ proinvasin I	→	"	antinvasin I
(c)	Proinvasin I	+ antinvasin II	→	"	proinvasin I

The over-all effect in this sequence of reactions will be that antinvasin I is left intact to fulfil its protective function against invasion by destroying hyaluronidase while antinvasin II is supporting this function through elimination of proinvasin I. The figures given in Table IX for the activity of antinvasin I against bacterial hyaluronidases are minimum values. These values depend not only on the relative proportion of hyaluronidase to proinvasin but also on the concentration of antinvasin II.

Variations in Concentration of Antinvasin I—The figures reported in Table IX for the concentration of antinvasin I represent the maximum values encountered in the course of this investigation. Considerable variations have been found, however, which are explained by the fact that invading organisms produce enzymes like proinvasin which destroy antinvasin.

Preliminary experiments with patients and healthy individuals seem

to indicate a striking correlation between physical condition of the individual and the concentration of this enzyme in plasma. In Table X a few isolated cases are summarized to illustrate this point. Hyaluronidase obtained from pneumococcus type VI was incubated for 10 minutes at 25° with 0.1 cc. of plasma and the activity of antinvasin I was determined from the amount of hyaluronidase destroyed. The results of this experiment are shown in Table X.

TABLE X

Antinvasin I in Plasma of Various Human Patients (Destruction of Hyaluronidase from Pneumococcus Type VI)

Diagnosis	Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0}\right]$
Healthy individuals (3 cases)	20
Cellulitis, right foot, general arteriosclerosis	11
Chronic ulcer of leg and cellulitis	9
Rheumatic fever	8
Paratyphoid carrier, recovered	6
Carcinoma of vulva, obesity, hypertension	5
Typhoid carrier	5

TABLE XI

Antinvasin I in Plasma of Various Human Patients (Destruction of Hyaluronidase from Bovine Testes)

Diagnosis	Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0}\right]$
Healthy individuals (10 cases)	6.3
Rheumatic fever	3.9
Cellulitis, general arteriosclerosis	3.2
Chronic ulcer of leg and cellulitis	2.6
Paratyphoid carrier, recovered	2.0
Type X pneumococcic pneumonia	1 8
Erysipelas of inguinal region	0.8

Objections may be raised to the results presented in Table X that specific antibodies are responsible for the observed destruction of pneumococcus hyaluronidase by human plasma. Protective antibodies to a variety of infections may be found in individuals who have never had a clinically recognizable case of the disease in question. This argument cannot be applied if the destruction of bovine testis hyaluronidase is used as an indicator of the antinvasin I concentration in plasma. It can be assumed that

human plasma does not contain antibodies against hyaluronidase of bovine testicle. 0.1 cc. of plasma of various individuals was incubated for 10 minutes at 25° with bovine testis hyaluronidase, and the activity of antinvasin I was measured in the test described in Table III. The results are summarized in Table XI.

Preliminary results, summarized in Tables X and XI, indicate a pronounced decrease of the antinvasin I concentration in the plasma of individuals with various disease conditions. A much more detailed investigation would be desirable to follow the concentration of these enzymes in the course of various infections.

The present investigation has not been extended far enough to indicate the significance of antinvasin I and II as therapeutic agents. It would be of interest to investigate this possibility not only in bacterial diseases but also in cases in which the toxic agent is a virus or comes from venomous organisms like poisonous reptiles.

SUMMARY

1. An enzyme has been found in normal blood plasma which, by destroying hyaluronidase, acts as an antiinvasion catalyst.

2. The properties of the new enzyme, antinvasin I, have been investigated as well as its incidence and distribution, and a method of assay is described.

3. The kinetics of the reaction have been studied, including the effects of temperature and pH on the activity and on the denaturation of the enzyme.

4. On the basis of these data the active principle can be differentiated from antibodies and it can be classified as an enzyme.

5. Antinvasin I has been found in the normal plasma of all mammals, birds, and fish investigated. It reacts with about equal velocity with hyaluronidase obtained from a number of sources.

6. The general occurrence and the unspecific reaction of antinvasin I further distinguish the enzyme from antibodies.

7. A pronounced decrease in the concentration of antinvasin I has been observed in individuals with various infections.

8. The wide distribution and reaction of antinvasin I with hyaluronidases from various sources suggest that the enzyme plays an essential rôle in the defense mechanism of the body by preventing invasion of certain bacteria and venoms.

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ON THE MECHANISM OF INVASION

II. PROINVASIN I, AN ENZYME IN PATHOGENIC BACTERIA AND IN VENOMS

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In Paper I (1) a scheme was presented to illustrate the interplay of various enzymes in processes of invasion and defense. An enzyme was described, antinvasin I, which had been found in plasma and which, by destroying hyaluronidase, acted as an antiinvasion catalyst. In the course of this investigation a second enzyme was observed in the culture medium of certain bacteria and in snake venoms. This enzyme, proinvasin I, is produced by the pathogenic organism simultaneously with hyaluronidase and acts by destroying antinvasin I, the defensive enzyme in plasma. Hyaluronidase, although normally inactivated by antinvasin I, is then left intact, since it is accompanied and protected by proinvasin I in amounts sufficiently large to cause destruction of antinvasin I. It can be assumed that proinvasin I will materially enhance the invasion of bacteria and venoms because, by eliminating antinvasin I, it permits the action of hyaluronidase to proceed unhindered.

In the present study the distribution of proinvasin I and its properties have been investigated. The reaction of the enzyme with antinvasin I in plasma of various species has been studied and an analytical test procedure devised for the quantitative determination of proinvasin I. The preparation of the test substances, as well as the viscometric method that is used for the determination of hyaluronidase and of antinvasin I, was described previously (1). The assay for proinvasin I essentially consists of measuring the amount of antinvasin I that has been inactivated by proinvasin I in the following system:

$$\text{Proinvasin I} \rightarrow \text{antinvasin I} \rightarrow \text{hyaluronidase} \rightarrow \text{polysaccharide}$$
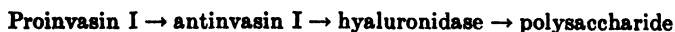
In the test procedure the antinvasin I of plasma is first incubated with the proinvasin of snake venom or bacteria. The amount of antinvasin I left intact after this time is determined by measuring its action on bovine hyaluronidase. Finally, the remaining hyaluronidase is measured by its

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activity in depolymerizing the polysaccharide. In its over-all effect, a rapid decrease in viscosity thus indicates high activity of proinvasin I.

Test for Proinvasin I (Proinvasin I of Snake Venom)

As a source of the enzyme, venom of the cottonmouth moccasin (*Agkistrodon piscivorus*) was used in the first set of experiments. While proinvasin I is present in the venom in high concentration, the amount of

TABLE I

Destruction of Antinvasin I of Human Plasma by Proinvasin I of Moccasin Venom

Ostwald viscosimeter, capillary diameter, 0.56 mm.; temperature, 25°; polysaccharide, 8 mg. per cc. of 0.02 M acetate buffer, pH 4.7; hyaluronidase from bovine testes, 0.50 mg. per cc. of water; source of antinvasin I, human plasma; source of proinvasin I, moccasin venom.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
0.2 M borate, pH 6.7, cc...	0.25	0.25	0.25	0.25	0.25	0.25
Water, cc......	0.60	0.60	0.50	0.50	0.40	0.40
Antinvasin I, cc....					0.10	0.10
Proinvasin I, mg...................				0.02		0.02

Solutions incubated 10 min., then combined with hyaluronidase

Hyaluronidase, cc..................			0.10	0.10	0.10	0.10

Solutions incubated 10 min., then combined with polysaccharide

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
0.5 M phosphate, pH 7.0, cc........	0.50	0.50	$\rightarrow$	$\rightarrow$	$\rightarrow$	$\rightarrow$
2 M NaCl, cc.	0.25	0.25				
Polysaccharide, cc.		1.00				
0.02 M acetate, pH 4.7, cc.	1.00					
Flow time, sec..	$t_2 = 39.2$	$t_1 = 92$				
Half life time, R_0, sec............			145	139		
" " " R, "					965	390
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$............					5.7	1.8
Destruction of antinvasin I by proinvasin I in 10 min. at 25°, %............						75

hyaluronidase is small; so that corrections for interference by hyaluronidase become negligible. Experimental details of the test are given in Table I, where the destruction of antinvasin I of human plasma by proinvasin I of moccasin venom has been investigated, according to the following procedures: Mixtures of proinvasin and antinvasin as well as the various controls were kept in a buffered solution for 10 minutes at 25° with occasional stirring. Antinvasin I, having been exposed to the action of

proinvasin I, was then allowed to react for 10 minutes at 25° with bovine testis hyaluronidase. The activity of the remaining hyaluronidase was then measured viscometrically.

In Experiments 1 and 2 the flow time is measured for the buffer and for the polysaccharide solution before the addition of hyaluronidase. Experiment 3 shows the activity of hyaluronidase. From a comparison of Experiments 3 and 4 it can be seen that the activity of hyaluronidase remains practically unchanged after its combination with proinvasin; *i.e.*, hyaluronidase does not react with proinvasin I. In Experiment 5 the activity of
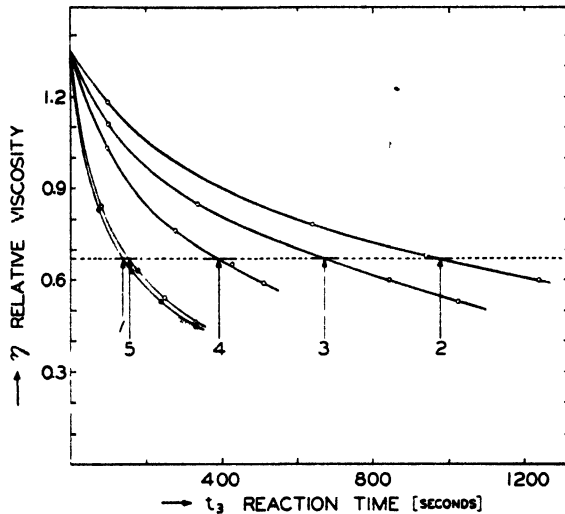
FIG. 1. Depolymerization of the polysaccharide as a function of proinvasin I. Curve 1, without plasma; Curve 2, with 0.1 cc. of plasma; Curves 3 to 5, with 0.1 cc. of plasma plus 0.01, 0.02, and 0.04 mg. of moccasin venom, respectively.

antinvasin I is obtained and, finally, by comparing Experiments 5 and 6 the destruction of antinvasin I by proinvasin I can be determined.

Effect of Various Concentrations of Proinvasin I—The destruction of antinvasin I as a function of proinvasin I was investigated by incubating a constant amount of antinvasin I of human plasma for 10 minutes at 25° with varying amounts of proinvasin of moccasin venom. In every other detail the experiments were conducted as described in Table I. Fig. 1 shows the rate at which depolymerization proceeds in the various experiments. It demonstrates clearly the pronounced effect of the two enzymes, antinvasin I and proinvasin I, on the breakdown of the substrate. These act indirectly and antagonistically to each other with the result that the rate of breakdown of the polysaccharide is altered.

A comparison of Curves 1 and 2 illustrates the destructive effect of ant-invasin I on hyaluronidase, which is indicated by the diminished rate of depolymerization. The progressive destruction of the activity of antin-vasin I by proinvasin I is demonstrated by comparing the slow rate of depolymerization in Experiment 2 with the increasing rates in Experiments 3, 4, and 5. In Fig. 2 the rate of destruction of antinvasin I is plotted as a function of the concentration of proinvasin I.

The crude venom of the cottonmouth moccasin is a very potent source of proinvasin I; as little as 0.04 mg. of the venom suffices to destroy 98 per cent of antinvasin I in 10 minutes under the conditions described in Table I. Hyaluronidase of venom, bacteria, etc., accompanied by such amounts
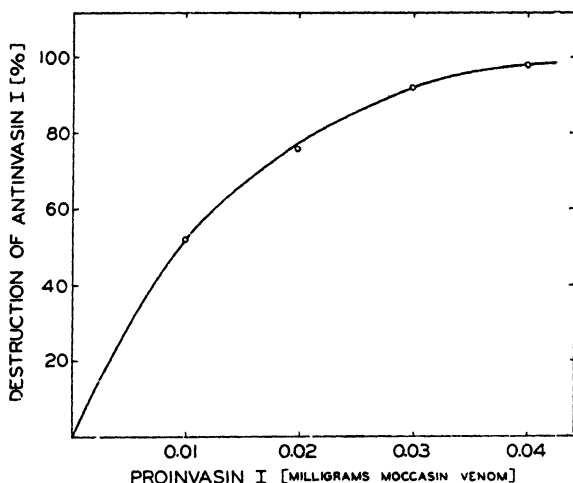
Fig. 2. Test for proinvasin I. Destruction of antinvasin I of human plasma by proinvasin I of moccasin venom.

of proinvasin I, is capable of playing its rôle as an invasive agent with maxi-mum efficiency because it is thus protected against destruction by antin-vasin I of plasma.

Effect of Time on Reaction, Proinvasin I-Antinvasin I—The high efficiency of proinvasin I can be demonstrated further by observing the speed with which it destroys antinvasin I. In Table II constant amounts of proinvasin I from moccasin venom, and of antinvasin I from human plasma, were combined. It will be seen that even at room temperature, and at small concentration of proinvasin I, rapid reaction occurs, resulting in the com-plete destruction of antinvasin I in the course of a few minutes.

Proinvasin I of Staphylococcus aureus—In the next experiment the reac-tion proinvasin I → antinvasin I was further investigated; but here the

proinvasin I was obtained from *Staphylococcus aureus* instead of snake venom. This preparation, which contains proinvasin I as well as hyaluronidase, was isolated as previously described (1). 600 cc. of the medium from a heavily seeded culture yielded 1.3 gm. of a crude enzyme. This preparation, although impure, contains sufficient proinvasin I in a sample of 5 mg. to destroy 65 per cent of antinvasin I in 10 minutes under the experimental conditions described. The activity of bacterial proinvasin I is shown in Table III; experimental details are similar to those described in Table I.

TABLE II

Destruction of Antinvasin I by Proinvasin I of Moccasin Venom As Function of Time

Experimental conditions as in Table I; temperature, 25°.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
0.2 M borate buffer, pH 6 7, *cc.*	0.25	0.25		
Water, *cc.*	0 40	0.40	→	→
Antinvasin I, *cc.*	0 10	0 10		
Proinvasin I, *mg.*		0.035		

Solutions incubated for various periods, then combined with hyaluronidase

		0 min.	1 min.	5 min.
Hyaluronidase, *cc*	0 10	0.10	0 10	0.10

Solutions incubated for 10 min., then combined with polysaccharide

0.5 M phosphate buffer, pH 7.0, *cc.*	0.50			
2 M NaCl, *cc*	0 25	→	→	→
Polysaccharide, *cc*	1 00			
Half life time, R_0, *sec.*	210	187	187	187
" " " R, "	1260	515	395	200
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$	5 0	1 8	1 1	0.07
Destruction of antinvasin I, %		70	81	98

The next experiments were performed to study the effect of various concentrations of proinvasin I on antinvasin I. In these studies proinvasin I prepared from cultures of different bacteria such as pneumococci, *Staphylococcus aureus*, and *Clostridium welchii* was employed. Fig. 3 represents a summary of these studies.

Three types of pathogenic bacteria and two venoms have been investigated and in each case the presence of proinvasin I was observed. It might be expected from this evidence that proinvasin I is widely distributed.

Reaction of Proinvasin I with Antinvasin I of Various Plasmas—Thus

TABLE III

Destruction of Antinvasin I of Human Plasma by Proinvasin I of Staphylococcus aureus

Source of proinvasin I, *Staphylococcus aureus;* source of antinvasin I, human plasma; source of hyaluronidase, bovine testes, 0.40 mg. per cc. of water; polysaccharide, 8 mg. per cc. of 0.02 M acetate buffer, pH 4.7.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4
0.2 M borate buffer, pH 6.7, *cc.*	0.25	0.25	0 25	0.25
Water, *cc*	0 50	0.45	0.50	0.45
Antinvasin I, *cc.* .			0.05		0.05
Proinvasin I, *mg.*				5 0	5.0

Incubated 10 min., then combined with hyaluronidase

Hyaluronidase, *cc.*	0.10	0.10	0 10	0.10

Incubated 10 min., then combined with polysaccharide

0.5 M phosphate, pH 7.0, *cc.*	0.50			
2 M NaCl, *cc* ..	0.25	$\rightarrow$	$\rightarrow$	$\rightarrow$
Polysaccharide, *cc.*	1 00			
Half life time, R_0, *sec.*	170		145	
" " " R, "		1180		450
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$	5 9		2.1	
Destruction of antinvasin I by proinvasin I in 10 min , %		65		
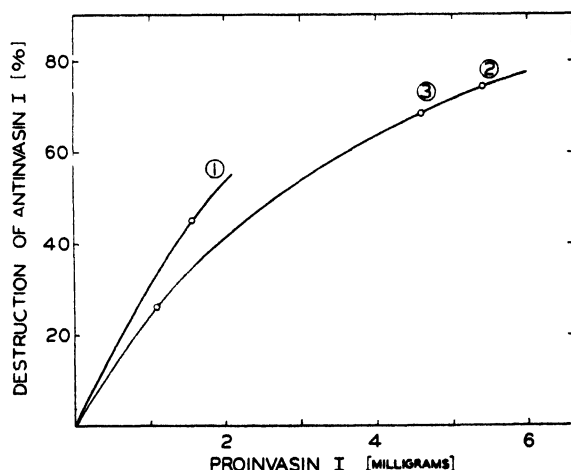
FIG. 3. Test for proinvasin I. Destruction of antinvasin I by proinvasin I from various bacteria. Curves 1 to 3, proinvasin I of pneumococcus type VI, *Staphylococcus aureus,* and *Clostridium welchii,* respectively.

far human plasma had been used as a source of antinvasin I to investigate the destruction of this enzyme by proinvasin I. Tables IV and V demonstrate that antinvasin I in plasma other than that of humans, *i.e.* mammals, birds, fish, is likewise destroyed by proinvasin I.

The activity of proinvasin I of snake venom was measured as described in Table I; *i.e.*, 0.1 cc. of the plasma was incubated for 10 minutes with 0.02 mg. of the venom, after which testis hyaluronidase was added and, finally, 10 minutes later, the decomposition of the polysaccharide was measured.

TABLE IV

Destruction of Antinvasin I of Various Plasmas by Proinvasin I of Moccasin Venom

Source of antinvasin I; plasma	Activity of antinvasin I		Destruction of antinvasin I by proinvasin I
	Without proinvasin I	0.02 mg. proinvasin I	
			per cent
Human	5 8	0.7	89
Chicken	3.4	1.2	66
Hog	4.3	1.0	78
Golden carp	1.5	1.0	33

TABLE V

Destruction of Antinvasin I of Various Plasmas by Proinvasin I of Staphylococcus aureus

Source of antinvasin I; plasma	Activity of antinvasin I		Destruction of antinvasin I by proinvasin I
	Without proinvasin I	5 mg. proinvasin I	
			per cent
Human, R. P.	8.9	3.4	65
" A. S.	6.5	5.9	9
Chicken	4.5	1.1	76
Hog	4.7	1.8	62
Carp	2.1	0.6	72

Table IV summarizes the effect of proinvasin I of moccasin venom on antinvasin I of four different plasmas.

The action of proinvasin I of *Staphylococcus aureus* on antinvasin I of various plasmas was determined under the conditions described in Table III. The results are presented in Table V. The findings summarized in Tables IV and V indicate that proinvasin I, derived either from bacteria or from venoms, is capable of reaction with antinvasin I in the plasma of all species investigated. This evidence indicates that proinvasin I, by. destroying the defensive enzyme in plasma, may play an essential rôle in the processes of invasion.

Protection of Hyaluronidase by Proinvasin I—It had been observed previously (1) that the velocity of reaction between antinvasin I and hyaluronidase varied greatly, depending on the origin of the hyaluronidase.   These variations can be explained now, when the concentrations of proinvasin as well as hyaluronidase are taken into account.   Table VI shows the relative

TABLE VI

Relative Proportion of Proinvasin I to Hyaluronidase in Various Sources

| Source of enzyme | Required for | | Proinvasin I |
	Hyaluronidase action*	Proinvasin I action†	Hyaluronidase
	mg	*mg*	
Pneumococcus type VI	1 25	1 75	0 7
Clostridium welchii	3 6	2 5	1 4
Staphylococcus aureus	9 0	2 5	3 6
Moccasin venom	0 10	0 009	11 0
Diamondback rattlesnake venom	0 06	0 05	1 2

* Concentration of hyaluronidase which will depolymerize 50 per cent of the polysaccharide in 10 minutes at 25°
† Concentration of proinvasin which will cause destruction of 50 per cent of antinvasin I in 10 minutes at 25°

TABLE VII

Variations in Destruction of Hyaluronidase of Testes, Bacteria, and Venom by Antinvasin I

Source of hyaluronidase*	Destruction of hyaluronidase†
	per cent
Testes	71
Pneumococcus type VI	64
Clostridium welchii	41
Staphylococcus aureus	26
Moccasin venom	0

* Concentration of hyaluronidase adjusted to depolymerize 50 per cent of the polysaccharide in 180 seconds = R_0
† Average values after reaction for 10 minutes at 25° with 0 1 cc of the following plasma: human, chicken, rabbit, beef, horse, and carp

concentration of these two invasion-promoting enzymes in preparations from various sources.   The extent to which hyaluronidase of various sources has been destroyed by antinvasin I under identical conditions is evident in Table VII.

By comparing the data in Tables VI and VII it will be seen that the relative proportion of proinvasin I to hyaluronidase determines the course

of events, and the following conclusions can be drawn. (1) With large amounts of proinvasin I, such as are present in moccasin venom, the destruction of antinvasin I takes place so rapidly that the reaction, antinvasin I-hyaluronidase, becomes negligible. In such a case hyaluronidase will not be destroyed by antinyasin I, and invasion can proceed. (2) With small amounts of proinvasin I (*e.g.* pneumococcus) the destruction of antinvasin I becomes negligible. The reaction, antinvasin I-hyaluronidase, therefore will proceed rapidly, and hyaluronidase produced by pneumococcus will be destroyed. Under these conditions antinvasin I in plasma would be capable of preventing invasion.

It is evident from this discussion that a knowledge of the rates at which the various bacterial enzymes are released may be of considerable use in predicting their action *in vivo*. The amount of hyaluronidase and of

TABLE VIII

Inactivation of Proinvasin I by Heat, Acid, and Alkali

Source of proinvasin I, moccasin venom.

Treatment (15 min.)		Destruction
Temperature	pH	
°C.		*per cent*
25	10.2	0
25	3.0	85
55	6.3	91

proinvasin I released into the medium by the bacteria after 8, 24, and 48 hours of growth was determined in a culture of *Staphylococcus aureus.* The absolute amount of the two enzymes increases with time. It was further noticed that the ratio of hyaluronidase to proinvasin I was not constant, but increased considerably with time. This fact may be taken as evidence that the enzymes appear in the solution not merely as a result of autolysis of the bacteria. It seems that proinvasin I becomes available first as a means of neutralizing the action of antinvasin I. Thereafter are released increasing amounts of hyaluronidase ("spreading factor"), the enzyme which facilitates invasion by decomposition of the mucoid ground substance of the connective tissue (2–5). Duran-Reynals (6) has shown that the spreading factor greatly enhances experimental bacterial and virus infection in the skin.

The experiments shown in Tables IV and V suggest that the destruction of antinvasin I by proinvasin I is a non-specific reaction of proinvasin of bacteria and venoms with antinvasin I, the enzyme in the plasma of all species investigated. Certain exceptions from this rule have been observed,

however, cases in which antinvasin I was destroyed much less than usual, e.g. in the plasma of patient A. S. (Table V). This was the first indication that normal plasma contains another enzyme, in addition to antinvasin I, which destroys proinvasin I and which acts therefore as a second anti-invasive factor. This enzyme, to which we will refer as antinvasin II, will be described in Paper III.

Stability of Proinvasin I against Denaturing Agents—A few preliminary data are accumulated in Table VIII to provide some indication as to the chemical nature of the enzyme, to serve as a guide for the future isolation, and to distinguish proinvasin I from other enzymes present in venom.

Some of the crude venoms have been found to be more potent than pure trypsin (7). Proinvasin I can be distinguished from trypsin by its response to acidic reaction. While trypsin can be kept (8) without appreciable loss for 24 hours at 30° and pH 3.0, proinvasin I in moccasin venom loses 85 per cent of its activity at this pH in 15 minutes at 25°. Lecithinase has been found in snake venoms but the heat stability of this enzyme compared with that of proinvasin I in moccasin venom indicates that the two enzymes are not identical: Lecithinase has been reported to be stable to prolonged boiling (9) while proinvasin I loses 91 per cent of its activity in 15 minutes at 55°. For the same reason proinvasin I cannot be identical with the thermostable, glycolysis-inhibiting substance observed in a number of snake venoms (10).

Other enzymes, such as 5-nucleotidases, phosphomono- and phospho-diesterases, have been found previously in venoms (11). The relation of the former enzyme to proinvasin I has not been investigated, but it seems certain that the "alkaline" type phosphoresterases are not identical with proinvasin I. Moccasin venom, which is a rich source of proinvasin I, is free of monoesterase, and the diesterase reacts much too slowly to be compared with proinvasin I.

SUMMARY

1. In the culture medium of certain pathogenic bacteria and in snake venoms a new enzyme has been found which will be referred to as "proinvasin I."

2. This enzyme is produced by the same organisms which produce hyaluronidase; its apparent function is the destruction of antinvasin I, the defense enzyme of plasma.

3. The relative proportion of the two enzymes, proinvasin I and hyaluronidase, varies greatly, depending on the source of the enzymes.

4. The relative proportion of these two enzymes will determine the course of the reaction; if only small amounts of proinvasin I are present, hyaluronidase is unprotected; it will be destroyed by antinvasin I, and invasion by

this route is prevented. With high concentration of proinvasin I, antinvasin I is rapidly destroyed and hyaluronidase, thus protected, is enabled to enhance invasion.

5. It can be assumed that proinvasin I, by protecting hyaluronidase from destruction by plasma enzyme (antinvasin I), contributes materially to the severity of infections.

6. An assay for proinvasin I has been described; some of the properties of the enzyme as well as its distribution in various sources have been investigated.

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ON THE MECHANISM OF INVASION

III. ANTINVASIN II, AN ENZYME IN PLASMA

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Two new enzymes have been described in previous papers (1).. The first, antinvasin I, which is part of the defense mechanism of the body, has been found in blood plasma. The second enzyme, proinvasin I, has been observed in the culture medium of pathogenic bacteria and in venoms; it constitutes apparently part of the mechanism of tissue invasion by bacteria and animal toxins. In the course of this investigation the presence of a third new enzyme was observed which will be described in the present paper. The function of this enzyme, which occurs in the plasma of all animals investigated, is the destruction of proinvasin I. It reacts therefore as an antiinvasive catalyst and we shall refer to it as antinvasin II.

Mechanism of Action of Antinvasin II

The activity of antinvasin II can be demonstrated by viscometric measurements, by the same method described previously for the determination of hyaluronidase, antinvasin I, and proinvasin I. The depolymerization of the polysaccharide under certain conditions is a function of the concentration of antinvasin II in the following sequence of reactions:

Antinvasin II → proinvasin I → antinvasin I → hyaluronidase → polysaccharide

Due to its intermediate position between antinvasins I and II, the action of proinvasin I will be determined largely by the relative proportion of antinvasin I to antinvasin II. For example, with large amounts of antinvasin I, such as are present in plasma of healthy individuals, proinvasin I will react with antinvasin I so fast that its reaction with antinvasin II can be neglected. Under these conditions antinvasin II cannot be demonstrated. Under certain conditions, however, *e.g.* in the plasma of patients with infections, the concentration of antinvasin I is so low that its rate of reaction with proinvasin I becomes negligible. In this case antinvasin II will react with proinvasin I, resulting in the destruction of proinvasin I. This is apparently the physiological rôle of antinvasin II: the destruction

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of proinvasin I, the enzyme employed by the invading organism.   This function of antinvasin II is of importance and it proceeds especially efficiently in those cases in which antinvasin I, the primary antiinvasive factor, has been depleted.

Antinvasin II seems to be a normal constituent of plasma and its presence can be demonstrated under various conditions.   (1) As already mentioned, it is present in plasma of individuals with various infections.   (2) Plasma of beef is an especially rich source of antinvasin II, and the presence of this enzyme can be observed easily because bovine plasma normally has a low antinvasin I content.   (3) Antinvasin II is more resistant to denaturation than antinvasin I.   Due to this fact, it has been possible to demonstrate the presence of antinvasin II also in every normal plasma.

<center>EXPERIMENTAL</center>

Experimental details have been given previously for the preparation of the test substances, for the arrangement of the tests, and for the calculation of the results (1).   Essentially the same method was applied in the present investigation on antinvasin II.   A few experiments summarized in Tables I and II will describe the procedure used for the determination of antinvasin II.

The experiments presented in Table I will illustrate some of the points mentioned in the introduction.   A comparison of Experiments 1 and 2 shows that proportionality exists between concentration and activity of antinvasin I.   From a comparison of Experiments 2 and 4 it becomes evident that the activity of antinvasin I is considerably diminished in the plasma of the patient with erysipelas of the inguinal region.   The experiment shows furthermore that proinvasin I from *Staphylococcus aureus* destroys in 10 minutes 60 per cent of antinvasin I in normal plasma (Experiments 2 and 3), but that it fails to destroy antinvasin I in the plasma of the patient (Experiments 4 and 5).

In Table II another set of experiments is presented to show that the apparent failure of proinvasin I to react with antinvasin I in the plasma of the patient is due to the destruction of proinvasin I by antinvasin II.

From the experiments described in Table II the following conclusions can be drawn: (1) The activity of antinvasin I in bovine plasma (Experiment 1) is 40 times lower than that found in normal human plasma (Experiment 2).   (2) Antinvasin I, in human plasma, is rapidly destroyed by proinvasin I of *Staphylococcus aureus* (Experiments 2 and 3), but the addition of 0.02 cc. of bovine plasma will afford almost complete protection for antinvasin I (Experiments 3 and 4).   From this fact it is concluded that bovine plasma contains an enzyme, antinvasin II, which is capable of de-

stroying proinvasin I. This destruction takes place so rapidly that further reaction of proinvasin I of *Staphylococcus aureus* with antinvasin I of human plasma is prevented. As the net result, antinvasin I maintains its activity, despite the presence of the bacterial enzyme (proinvasin I). (3) No direct reaction takes place between antinvasin II and hyaluronidase (Experiment 1) and no reaction has been observed between antinvasins I and II (Experiments 2 and 5).

<div align="center">TABLE I</div>

Reaction of Proinvasin I with Antinvasin I of Various Plasmas (Proinvasin I, from Staphylococcus aureus)

Plasma, R. P., from a healthy individual; plasma, A. F., from a patient with erysipelas; hyaluronidase, from bovine testes (0.4 mg. per cc.); polysaccharide, 8 mg. per cc. of 0.02 M acetate buffer, pH 4.7; temperature, 25°.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
0.2 M borate buffer, pH 6.7, *cc*	0.25	→	→	→	→
Water, *cc*	0.42				
Plasma, R. P. (antinvasin I), *cc*	0.04	0.08	0.08		
" A. F. (" II), *cc*				0.08	0.08
Proinvasin I, *mg*			5.0		5.0

<div align="center">Solutions incubated 10 min., then combined with hyaluronidase</div>

Hyaluronidase, *cc*	0.10	0.10	0.10	0.10	0.10

<div align="center">Solutions incubated 10 min., then combined with polysaccharide</div>

0.5 M phosphate, pH 7.0, *cc*	0.50	→	→	→	→
2 M NaCl, *cc*	0.25				
Polysaccharide, *cc*	1.00				
Half life time, R_0, *sec*	170	170	135	170	135
" " " R, "	810	1420	545	525	490
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$	3.8	7.4	3.0	2.1	2.6
Destruction of antinvasin I by proinvasin I in 10 min. at 25°, %	60				0

The next experiment describes the destruction of proinvasin I of *Staphylococcus aureus* at various concentrations of antinvasin II, and for various sources of this enzyme. The experimental conditions are the same as described in Tables I and II; the results are shown in Fig. 1.

An inspection of the results, plotted in Fig. 1, reveals that as little as 0.006 cc. of bovine plasma suffices to destroy in 10 minutes at 25° 50 per cent of the proinvasin I present. The specimen of human plasma was found to be approximately 6 times less effective in destroying proinvasin I.

TABLE II ❄

Destruction of Proinvasin I of Staphylococcus aureus by Antinvasin II of Bovine Plasma

Source of antinvasin I, normal human plasma; of antinvasin II, bovine plasma; of hyaluronidase, bovine testes (0.40 mg. per cc.) temperature, 25°.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
0.2 M borate buffer, pH 6.7, *cc.*	0.25	→	→	→	→
Water, *cc.*	0.52				
Antinvasin I, *cc.*		0.08	0.08	0.08	0.08
" II, *cc.*	0.08			0.02	0.02
Proinvasin I, *mg.*			5.0	5.0	

Solutions incubated 10 min., then combined with hyaluronidase

Hyaluronidase, *cc.*	0.10	0.10	0.10	0.10	0.10

Solutions incubated 10 min., then combined with polysaccharide

0.5 M phosphate, pH 7.0, *cc.*	0.50				
2 M NaCl, *cc.*	0.25				
Polysaccharide, *cc.*	1.00 (8 mg.)				
Half life time, R_0, *sec.*	170	170	135	135	170
" " " R, "	200	1420	545	1040	1430
Activity of antinvasin I $\left[\dot{A} = \dfrac{R - R_0}{R_0}\right]$.	0.18	7.4	3.0	6.7	7.4
Destruction of antinvasin I by proinvasin I, %			60	9	
" " proinvasin I " antinvasin II, %				88	
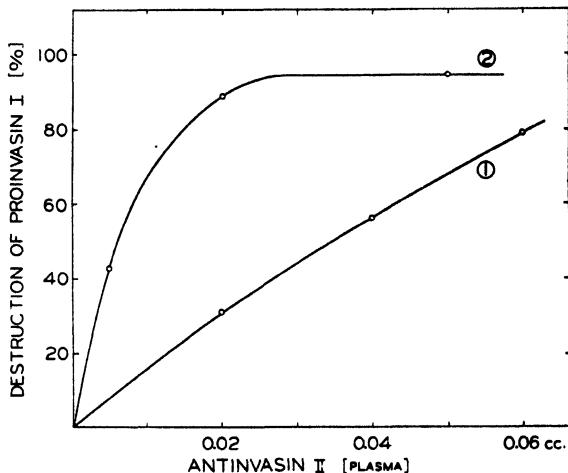
FIG. 1. Destruction of proinvasin I as a function of antinvasin II (test for antinvasin II). Curve 1, antinvasin II in plasma of patient with erysipelas; Curve 2, antinvasin II in plasma of beef.

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The latter value, for reasons mentioned in the introduction, depends considerably on the condition of the patient (relative proportion of antinvasin I to antinvasin II).

Stability of Antinvasin II against Denaturation—Antinvasin II, in human plasma, had been found so far only in certain patients and it seemed of interest to determine its presence also in normal individuals. As a prerequisite, in testing for antinvasin II, it is essential to eliminate antinvasin I from the plasma. Experiments were performed therefore to investigate the stability of antinvasin II under various conditions. On the basis of this information it was possible to remove antinvasin I from normal plasma and to test for antinvasin II.

Samples of human plasma were adjusted by the addition of NaOH or HCl to the conditions described in Table III, then incubated, and finally neutralized. The remaining activity of antinvasin II was measured, as described in Table II and Fig. 1, and compared with that of an untreated

TABLE III

Antinvasin II in Human Plasma, Effect of Acid, Alkali, and Heat (Comparison with Antinvasin I)

Treatment (15 min.)		Inactivation	
Temperature	pH	Of antinvasin II	Of antinvasin I
°C.		per cent	per cent
25	10.1	0	20
25	3.0	16	87
55	7.8	0	84
60	7.8	16	100

aliquot. The results of these experiments are summarized in Table III and a comparison is made of the action of various denaturing agents on antinvasin I and II.

Antinvasin II, the enzyme which destroys proinvasin I, is much more résistant to denaturation than antinvasin I, the enzyme which destroys hyaluronidase. Antinvasin I activity is abolished by incubation of plasma for 15 minutes at 60°, while antinvasin II essentially retains its activity under the same conditions.

Reaction of Antinvasin II of Human Plasma with Proinvasin I of Moccasin Venom—The experiments described in Table IV were undertaken, first, to demonstrate the presence of antinvasin II in normal human plasma and, secondly, to show that this enzyme is capable of destroying proinvasin I of snake venom as effectively as that of bacteria. The latter observation is of interest because it indicates a non-specific reaction of antinvasin II with proinvasin I obtained from widely different sources. On this basis it is concluded that antinvasin II does not react as an antibody.

The action of antinvasin II, directed against proinvasin I of various invading organisms, may possibly be of practical interest and it would be desirable to investigate the rôle of antinvasin II as a therapeutic agent in the treatment of bacterial infections and of snake-bites.

The destruction of hyaluronidase by antinvasin I is evident from Experiments 1 and 2; the destruction of antinvasin I by proinvasin I is illustrated by comparing Experiments 2 and 4, and finally, the destruction of proin-

TABLE IV

Destruction of Proinvasin I of Moccasin Venom by Antinvasin II of Human Plasma

Source of antinvasin I, normal human plasma; of antinvasin II, normal human plasma incubated 15 min. at 60°; of hyaluronidase, bovine testes (0.40 mg. per cc.).

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5
0.2 M borate buffer, pH 6.7, cc.	0 25	0.25	0.25	0 25	0 25
Water, cc	0 50	0.40	0.30	0 40	0 30
Antinvasin I, cc.		0.10	0.10	0 10	0 10
" II, cc.				0.10	0 10
Proinvasin I, mg.				0 02	0.02
Solutions incubated 10 min., then combined with hyaluronidase					
Hyaluronidase, cc.	0.10	→	→	→	→
Solutions incubated 10 min., then combined with polysaccharide					
0.5 M phosphate, pH 7.0, cc.	0.50				
2 M NaCl, cc.	0.25	→	→	→	→
Polysaccharide, cc	1 00 (8 mg.)				
Half life time, R_0, sec	270	270	270	245	245
" " " R, "		1840	1840	410	1350
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$	..	5.8	5.8	0.7	4.5
Destruction of antinvasin I by proinvasin I, %.		..		88	22
" " proinvasin I " antinvasin II, %		.			92

vasin I by antinvasin II becomes apparent by confronting the results of Experiments 4 and 5.

Antinvasin II has no direct action on antinvasin I and it does not react directly with hyaluronidase (Experiment 3).

Reaction of Antinvasin II of Various Plasmas with Proinvasin I of Moccasin Venom—The experiments reported in Table IV have been extended in Table V to include plasmas of five different species. In every case it was possible to demonstrate the presence of antinvasin II by the rapid destruction of proinvasin I. This is taken as evidence that antinvasin II

TABLE V

Destruction of Proinvasin I of Moccasin Venom by Antinvasin II of Various Plasmas

Source of antinvasin I, normal human plasma; of antinvasin II, plasma, of human, beef, hog, chicken, and carp, incubated 15 minutes at 60°; experimental details are identical with those in Table IV.

	Experiment 1	Experiment 2	Experiment 3	Experiment 4	Experiment 5	Experiment 6
0.2 M borate buffer, pH 6 7, cc.	0.25	0.25				
Water, cc.	0 40	0.30				
Antinvasin I, cc.	0 10	0.10				
" II, cc.		0 10 (Human)	Beef ↑	Hog ↑	Chicken ↑	Carp ↑
Proinvasin I, mg.	0.02	0.02				
Solutions incubated 10 min., then combined with hyaluronidase						
Hyaluronidase, cc.	0.10	↑	↑	↑	↑	↑
Solutions incubated 10 min., then combined with polysaccharide						
0.5 M phosphate, pH 7.0, cc.	0.50	↑	↑	↑	↑	↑
2 M NaCl, cc.	0.25					
Polysaccharide, cc.	1 00 (8 mg.)					
Half life time, R_0, sec.	245	245	245	245	245	245
" " R, "	410	1420	1130	1260	1260	1235
Activity of antinvasin I $\left[A = \dfrac{R - R_0}{R_0} \right]$	0.7	4.8	3.6	4.1	4.1	4.0
Destruction of antinvasin I by proinvasin I, %	88	17	39	29	29	31
" proinvasin I by antinvasin II, %		94	81	88	88	87

is a normal constituent of plasma in mammals, birds, and fish. It lends further support to the statement made previously that antinvasin II is not an antibody. (These animals had not been exposed to snake venom.)

It has been proposed (2, 3) to use the presence of hyaluronidase as indicator for the presence of certain pathogenic bacteria in wounds. Inconsistent results obtained with this method have been reported (4), which may be explained in terms of the reactions of these new enzymes. (a) Hyaluronidase cannot be demonstrated in the presence of plasma, because antinvasin I in plasma will cause the rapid destruction of hyaluronidase. (b) In a mixed infection of wounds, containing for example both pneumococcus type VI and *Staphylococcus aureus*, hyaluronidase may be demonstrated because proinvasin I, the enzyme produced by pathogenic bacteria, will rapidly destroy antinvasin I. Under these conditions hyaluronidase may accumulate. (c) However, even in a mixed infection, hyaluronidase may

TABLE VI

Destruction of Proinvasin I by Antinvasin II (Antinvasin II from Six Sources, Proinvasin I from Two Sources)

Source of antinvasin II;* plasma	Destruction of proinvasin I	
	Of moccasin venom	Of *Staphylococcus aureus*
	per cent	*per cent*
Human	94	27
"	92	44
Beef	81	75
Hog	88	0
Chicken	88	0
Carp	87	0

* Each plasma had been incubated for 15 minutes at 60° to eliminate antinvasin I.

disappear in the following sequence of enzymatic reactions: Antinvasin II, which is present in certain human plasmas in relatively large amounts, will destroy proinvasin I. Antinvasin I, thereby protected from the action of proinvasin I, is now capable of reacting with hyaluronidase.

Indication for Additional Factors in Process of Invasion (Proinvasin II and Antinvasin III)—The last experiment (Table VI) is presented as a preliminary study, which seems to indicate the participation of two more constituents in this system of invading and defending enzymes.

Plasmas of various species were investigated (Table VI) for their concentration of antinvasin II. All six specimens were found to be very active, as judged by their ability to destroy proinvasin I of moccasin venom. However, when aliquots of these plasmas were tested against proinvasin I from *Staphylococcus aureus*, different results were obtained: Antinvasin

II of hog, chicken, carp, and human plasma either proved to be completely unable to destroy proinvasin I of *S. aureus*, or its destructive activity on the latter was much less than on proinvasin I of moccasin venom. Antinvasin II is an unspecific enzyme, not an antibody; its failure to destroy proinvasin I indicates therefore the presence of an interfering factor in the enzyme preparation from *S. aureus*. We shall refer to this factor, presumably an enzyme, tentatively as "proinvasin II," to imply that by its action the invasion of bacteria will be promoted. A comparison of the results in Table VI shows that proinvasin II, in the enzyme preparation from *S. aureus*, has reduced materially the activity of antinvasin II in human plasma and has completely inactivated antinvasin II in the plasma of hog, chicken, and carp, as indicated by its failure to destroy proinvasin I. With antinvasin II removed, proinvasin I can act to promote bacterial invasion, as previously described. The enzyme system involved in the process of invasion seems to be more complex in bacteria than in venom; proinvasin II was observed only in preparations from *S. aureus*, not in moccasin venom. The existence of proinvasin II in the enzyme preparation from *S. aureus* was postulated after complete destruction of antinvasin II had been observed in the plasma of hog, chicken, and carp. An inspection of the data in Table VI reveals that antinvasin II had been much less destroyed under equal conditions in human and in bovine plasma. This apparent discrepancy can be explained by assuming tentatively the presence in plasma of another enzyme, "antinvasin III," which would act by destroying proinvasin II of *S. aureus*. The course of events is then determined by the concentration of antinvasin III in the following chain of reactions:

Antinvasin III → proinvasin II → antinvasin II → proinvasin I →
                                        antinvasin I → hyaluronidase → polysaccharide
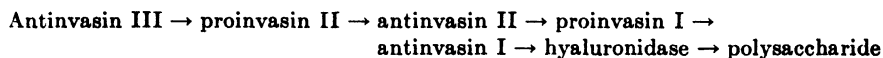
A relatively high concentration of antinvasin III seems to occur in human and in bovine plasma, which results directly in destruction of proinvasin II and which leads indirectly to the destruction of proinvasin I and hyaluronidase.

It should be emphasized that the conclusion reached with respect to the last two enzymes, proinvasin II and antinvasin III, is considered preliminary. Separation and purification of the various components will be required to continue the study of this complex system in greater detail.

<center>SUMMARY</center>

1. Evidence is presented for the occurrence of a new enzyme in plasma, designated as "antinvasin II."

2. The action of antinvasin II involves the destruction of proinvasin I,

the enzyme released by certain invading organisms.   It is assumed that antinvasin II, due to this action, functions as a catalyst counteracting invasion and spreading of certain pathogenic bacteria and venoms.

3. 'A procedure for the assay of antinvasin II is described, which allows the study of its properties and which makes it possible to demonstrate its presence in various plasmas, including that of patients.

4. Based on preliminary evidence, the participation of two more enzymes may be predicted in this system, one, "proinvasin II," postulated to occur in *Staphylococcus aureus*, the other, "antinvasin III," presumably occurring in plasma.

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RIBONUCLEINASE

III. THE BEHAVIOR OF COPPER AND CALCIUM IN THE PURIFICATION OF NUCLEIC ACID AND THE EFFECT OF THESE AND OTHER REAGENTS ON THE ACTIVITY OF RIBONUCLEINASE

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The reactivity of most commercial samples of nucleic acid with ribonucleinase was shown (1) to be approximately doubled by precipitation with 5 volumes of acetic acid, an improvement which was due to the elimination of mononucleotides. In one instance, however, removal of the mononucleotides in this manner did not increase the activity. Copper has been reported (2) to be inhibitory to ribonucleinase and, since copper vessels are occasionally used in industry, the presence of this metal was suspected. Further investigation has confirmed the inhibitory effect of copper on ribonucleinase and shown that copper was present in the nucleic acid and that it accompanied almost quantitatively the acetic acid-insoluble high polymer nucleic acid. For this reason, the activity of the ribonucleinase with the precipitated nucleic acid was less than with the crude nucleic acid.

With some samples of nucleic acid, added copper was not inhibitory until a certain concentration was exceeded. Further experiments suggested that the anti-copper factor might be an —SH compound. Since copper is usually regarded as inhibitory to enzymes because of its reactivity with essential —SH groups, other reagents that react with —SH groups, oxidizing agents, alkylating agents, etc., were employed with ribonucleinase. Several of these reagents were inhibitory.

Calcium, variable amounts of which are contained in the crude nucleic acids, was also found to be concentrated by precipitation with acetic acid, but calcium was not inhibitory to ribonucleinase. Copper and zinc were found to be much more inhibitory than numerous other metals studied.

Procedure

The activity of the ribonucleinase was determined by the manometric method (2), as in previous studies (1, 3). Crystalline ribonucleinase was used and the substrate ribonucleic acid was purified by precipitating with 5 volumes of glacial acetic acid. The metallic substances whose effects on ribonucleinase were being studied were placed in the main part of the Warburg flasks with the nucleic acid and sodium bicarbonate, and the ribonucleinase was introduced from the side arm after equilibrium had been reached. The other substances under study were added to the enzyme in

the side arm or in the main part of the flask and the nucleic acid was placed in the side arm. This procedure afforded time for interaction with the enzyme before it was mixed with the substrate.

The data confirming the inhibitory effect of copper (2) are shown in Fig. 1, Curve A. In view of this effect the copper contents of several nucleic acids were determined. 0.5 gm. samples of nucleic acid were digested with 40 cc. of nitric acid and 10 cc. of sulfuric acid for about 10 hours; the digests were heated to dryness, dissolved in 1:1 HCl, dried again, and redissolved in 0.5 N HCl. These solutions were partly neutralized to the point at which a precipitate, presumably calcium phosphate, started to form. The so-
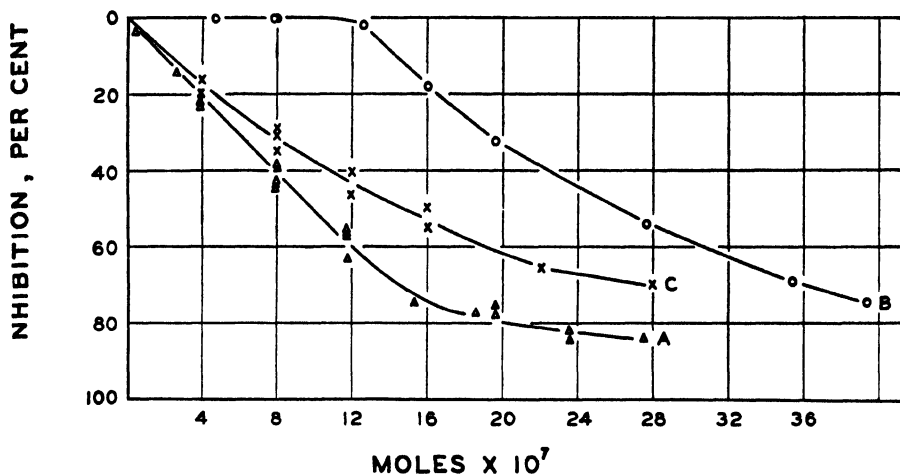
FIG. 1. The effect of copper and zinc on the activity of ribonucleinase. 140 mg. of nucleic acid were used as the substrate. The number of moles of copper or zinc indicated are contained in 3.5 cc. Curve A, effect of copper with nucleic acid, Sample EA_2. Curve B, effect of copper with nucleic acid, Sample EA_1. Curve C, effect of zinc with nucleic acid, Sample EA_2. Like data were obtained for Curves A and B when the metals were added to the enzyme before mixing with the substrate.

lutions were clarified by centrifuging, the supernatant fluids adjusted to a volume of 25 cc., and 10 cc. aliquots taken for copper analysis by the thiocyanate method (4).

The copper found in several commercial samples of nucleic acid before and after precipitation with acetic acid is recorded in Table I, together with the reactivities of the nucleic acids with ribonucleinase. The samples (P and E_1) that contained a small amount of copper on precipitation showed a good increase in activity,[1] whereas those that contained larger amounts of

[1] Presumably the small amount of copper present was concentrated by precipitation and would have been inhibitory if the increase in reactivity due to the removal of mononucleotides had not predominated.

copper (Samples E_2 and S) showed negligible increase in activity. With the nucleic acids, Samples E_2 and S, a 2.6- to 2.8-fold increase in the copper content resulted from precipitation. Since the amount of nucleic acid recovered in this step was about 25 per cent, a 4-fold increase in the copper would be expected if all of the copper had accompanied the precipitate; according to the above data about 67 per cent of the copper accompanied the high polymer nucleic acid on precipitation. This was shown not to be a peculiar property of the copper contained in the nucleic acids, by adding copper sulfate to Sample E_2 before precipitation to make the total copper concentration 53 γ per 100 mg. The nucleic acid after precipitation (25 per cent was recovered again) contained 131 γ of copper per 100 mg. or

TABLE I

Certain Properties of Nucleic Acid before and after Precipitation with Glacial Acetic Acid

Sample* of nucleic acid	Nucleic acid recovered in pptn.	Reactivity with ribo-nucleinase†	Copper content	Calcium content	Ash content
	per cent	*c.mm. per 1 γ per hr.*	γ *per 100 mg.*	*per cent*	*per cent*
P, unprecipitated		14.5	12	0.64	11.6
" precipitated	40	26.9		1.19	14.0
E_1, unprecipitated		16.1	14		
" precipitated	64	24.0			
E_2, unprecipitated		14.2	21	0.05	11.6
" precipitated	25	18.9	59	0.18	11.9
S, unprecipitated		15.2	34	0.15	14.7
" precipitated	22	14.0	88	0.47	12.5

* These samples were obtained commercially: P, Pfanstiehl; E, Eastman; S, Schwarz.

† In these measurements 180 mg. of the nucleic acid were used as the substrate. The activity is expressed as c.mm. of CO_2 evolved per microgram of ribonucleinase per hour.

63 per cent of the copper accompanied the precipitated nucleic acid. This increase in the copper content is sufficient to cancel out the increase in activity expected from the elimination of mononucleotides.

In the copper analyses it was noted that if the digests were completely neutralized the precipitate which formed, thought to be calcium phosphate, was larger in the precipitated samples of nucleic acid than in the unprecipitated. To make the observation quantitative the digests described above were analyzed for calcium. The digests were adjusted to pH 3 to 4, a pH at which little or no calcium phosphate precipitated,[2] the calcium pre-

[2] The appearance or non-appearance of this precipitate was not considered important since it has been shown (5) that calcium phosphate readily changes to calcium oxalate in the presence of ammonium oxalate.

cipitated with ammonium oxalate, and the precipitate of calcium oxalate titrated with standard permanganate. The results obtained are shown in Table I. Comparison of the calcium content of the unprecipitated and precipitated nucleic acids, corrected for the amount of nucleic acid recovered, shows that the calcium accompanied almost quantitatively the high polymer nucleic acid. Other divalent ions may behave in the same way. The ash content (Table I) may be determined by the ratio of monovalent and divalent ions. When the calcium content was high (Sample P), the ash content of the precipitated nucleic acid was increased; when the calcium content was low (Samples E_2 and S) the ash content remained the same or decreased. The presence of calcium apparently had no effect on the reactivity of the ribonucleinase with the nucleic acid since removal of the calcium with sodium oxalate did not affect the activity.

Ribonucleinase was inhibited by copper with three samples (P, EA_2, and EA_3) of nucleic acid, as shown by Curve A (Sample EA_2) of Fig. 1. With other samples of nucleic acid studied small concentrations of copp r did not inhibit ribonucleinase. One of these (Sample EA_1) was studied over a wide range of copper concentrations (Curve B, Fig. 1) and it was found that after 1.1×10^{-6} mole of copper was exceeded, the inhibition curve had about the same shape as Curve A.[3] It should be noted that zinc has been about equally inhibitory to ribonucleinase with no zone of no inhibition with all nucleic acids studied (Curve C, Fig. 1).

Sulfhydryl compounds and pyrophosphate were considered as substances that might be interfering with the action of copper. It was found that an excess of cysteine (2.0×10^{-6} mole) prevented the action of 1.6×10^{-6} mole of copper with nucleic acid Sample EA_2 but that the inhibition of the same amount of zinc was decreased only about 25 per cent. Since this experiment suggested that —SH compounds were involved, an attempt. was made to destroy the —SH compounds in a sample of nucleic acid (Sample PL) by oxidation with H_2O_2.[4] This treatment did not change the reactivity of the nucleic acid with ribonucleinase and decreased the zone of low inhibition only slightly (decreased from 1.6 to 1.2×10^{-7} mole). The slope of the inhibition curve was not changed.

Pyrophosphate (2.0×10^{-6} mole) decreased the inhibitory effect of zinc (1.6×10^{-6} mole) on ribonucleinase about 30 per cent and had little or no

[3] The identity of curves was not found with all nucleic acids; the slope of Sample P was slightly less than that of Sample EA_2 (this difference was shown by zinc with the same nucleic acids) and the curve for Sample PL ("pool"), which contained considerable anticopper substance, was very flat. The significance of the different slopes is not apparent.

[4] 100 cc. of 10 per cent nucleic acid at pH 7.4 were slowly treated with 15 cc. of 3.0 per cent H_2O_2 at about 80°. This treatment bleached the solution from a dark brown to a straw color. The nucleic acid after standing 18 hours at 7° was precipitated with 5 volumes of glacial acetic acid in the usual way.

influence on the action of copper. This was not studied in greater detail because this small amount of pyrophosphate precipitated some of the nucleic acid and was slightly inhibitory (about 8 per cent). These experiments were not conclusive, but it appears probable that an —SH compound is involved in the interference with the action of copper on ribonucleinase encountered with some samples of nucleic acid.

The copper and zinc enzyme-inhibition curves (Curves A, B, and C) in Fig. 1 are essentially hyperbolic. In the case of Curve B the incomplete solubility of the basic carbonate of the metal, formed from the bicarbonate required in the test system, may be a contributing factor, although it was observed that the nucleic acid increased the solubility of the copper. This is not a factor with inhibition Curves A and C; the shape of these curves is indicative of the formation of a dissociable compound between the metals and the enzyme (2).

Other metals were tested on ribonucleinase in the usual way but none had very much effect compared with copper and zinc. Silver $(1.0 \times 10^{-6}$ mole, calculated from the solubility of the carbonate) caused less than 10 per cent inhibition; mercuric chloride $(2.0 \times 10^{-6}$ mole) had a negligible effect. Cobaltous, cadmium, and ferric chlorides $(2.0 \times 10^{-6}$ mole) had a negligible effect on ribonucleinase; the same concentration of nickelous chloride exerted about 10 per cent inhibition. Phenylmercuric acetate tested in this manner had a negligible effect, but when in contact with the enzyme for about 30 minutes before mixing with the substrate, 2×10^{-6} mole of this compound produced 25 per cent inhibition. Saturation of the test system with this compound gave 60 per cent inhibition.

Oxidizing agents were tested because of their expected reaction with —SH groups which might be in the ribonucleinase molecule. The effect of arsenite and selenite on the activity of ribonucleinase was negligible.[5] Maleic acid was not inhibitory. Saturation of the test system with cystine had no effect on the activity of the ribonucleinase.

Iodoacetate and iodoacetamide did not inhibit when placed with nucleic acid during the attainment of equilibrium, but were inhibitory (Fig. 2) when added to the ribonucleinase. These compounds are relatively slow in their action (6, 7).

<div align="center">DISCUSSION</div>

Most enzyme studies have been handicapped by the use of an impure enzyme, whereas in the present studies the enzyme is pure but unfortunately the substrate is impure. The data in Table I for the copper and the calcium contents of unprecipitated and precipitated nucleic acid in-

[5] These and subsequent data have been corrected for the ██O₂ retained by the buffering capacity of the substances under test (1) and the experiments were performed with nucleic acids that contained no anti-copper factor.

dicate one of the difficulties that will be encountered in completely purifying nucleic acid and also serve to characterize the available material. Precipitation with 5 volumes of glacial acetic acid serves principally to eliminate the low polymer nucleic acid, the tetra- and mononucleotides (1); accordingly it can be concluded that the divalent copper and calcium are preferentially bound by the high polymer, multivalent fraction of the nucleic acid.

Another component of interest in the nucleic acid is the substance which neutralizes the effect of copper to some extent. It may be a sulfhydryl compound but, if so, it is difficult to understand why it was not quantitatively destroyed by H_2O_2.
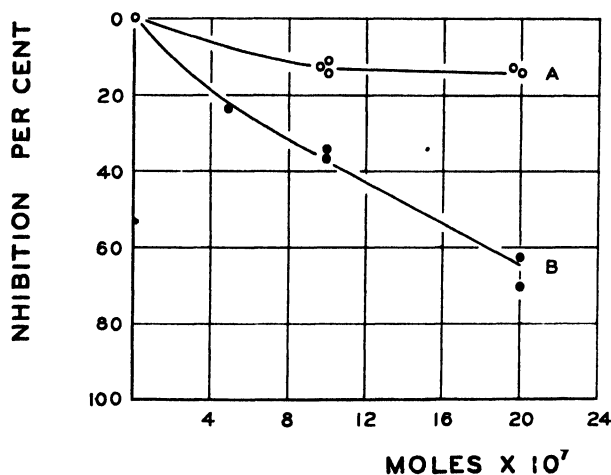
Fig. 2. The effect of iodoacetamide and iodoacetic acid on ribonucleinase. Curve A, iodoacetamide; Curve B, iodoacetate.

Copper is the most inhibitory substance for ribonucleinase that has been encountered; even so the amounts giving inhibition are large compared with the amount of ribonucleinase used in each experiment, namely 30 γ, which equals 2.3×10^{-9} mole (8). This is a reflection of the degree of association between enzyme and copper. The hyperbolic inhibition curve indicates that a dissociable compound with the enzyme is involved. The inhibitory effect of silver on urease gives a curve (9) of this type, but in this case the degree of association is much greater, much less silver being required for a comparable degree of inactivation. Silver, mercury, and other metals were not inhibitory to ribonucleinase under the same conditions.

The relative effects of iodoacetamide and iodoacetate are contrary to the usual experience with these reagents; the former reacts more rapidly with —SH compounds (7) and is more inhibitory to enzymes (7, 10); however, the iodoacetate is the more inhibitory to fermentation by living yeast cells

and by a cell-free yeast extract (7). The compounds used in the present experiments gave no iodine reaction with starch and reacted with cysteine to the same extent, as measured by the evolution of CO_2 from 0.1 M $NaHCO_3$ by the HI formed. Iodide, present to some extent in both the compounds, was not inhibitory to ribonucleinase even when 1.0×10^{-4} mole was used. As a further check on the reagents, their action on urease in jack bean meal was determined manometrically (7). The results reported by others (7) were obtained; the iodoacetamide was almost completely inhibitory and the iodoacetic acid only slightly inhibitory. It was considered that because of the basic nature of the ribonucleinase (isoelectric point = 7.8 (8)) an inactive salt might be formed with the iodoacetic acid, but this seems unlikely since trichloroacetate had a negligible effect on the ribonucleinase. A basic protein may be more reactive with the negatively charged iodoacetate than with the iodoacetamide and, without information on this point, it cannot be concluded that groups other than —SH are involved in the inactivation of ribonucleinase.

SUMMARY

Copper and calcium, both present in commercial nucleic acids, accompanied the insoluble high polymer fraction when a nucleic acid solution was precipitated with acetic acid. Copper inhibited ribonucleinase, and when the amount of copper present in the crude nucleic acid was relatively large it became noticeably inhibitory when the precipitated nucleic acid was used as the substrate for ribonucleinase. The presence of calcium apparently had no effect on ribonucleinase. A substance was present in some lots of nucleic acid which prevented the inhibition of ribonucleinase by copper until a certain concentration of copper was exceeded. Copper and zinc apparently inhibit by the formation of an inactive dissociable complex with the enzyme. Zinc was almost as inhibitory as copper; other metals tested had a negligible effect; phenylmercuric acetate was inhibitory. Iodoacetate and iodoacetamide, —SH reagents, also inhibited ribonucleinase. Contrary to the action of each on other enzymes, iodoacetic acid was more inhibitory than iodoacetamide. Arsenite, selenite, cystine, and maleic acid had no effect on ribonucleinase.

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RIBONUCLEINASE

IV. HYDROLYSIS OF RIBONUCLEIC ACID BY RIBONUCLEINASE AND BY SODIUM HYDROXIDE

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In his thorough study of ribonucleinase Kunitz (1) observed that ribonucleic acid even after long digestion with an excess of enzyme was still 10 to 15 per cent precipitable with glacial acetic acid and 60 per cent precipitable with uranium acetate in trichloroacetic acid. It was surmised that this might be due to the presence of some modified nucleic acid which cannot be attacked by the enzyme. Loring and Carpenter (2) pointed out that the titration data of Allen and Eiler (3) were in agreement with the observation of Kunitz. Loring (4) subsequently confirmed and extended these findings and concluded that ribonucleic acid must contain at least two different types of linkages, one of which is labile and one of which is resistant to the action of ribonucleinase. Our own studies (5) had shown that mononucleotides were inhibitory to ribonucleinase, and it was thought that the accumulation of mononucleotides might account for the incomplete digestion of ribonucleic acid. Studies reported herein in detail show that this cannot be the explanation. To throw further light on this phenomenon the course of hydrolysis of ribonucleic acid by enzyme and by sodium hydroxide, measured by precipitation with uranium chloride in trichloroacetic acid, has been studied, as well as the action of the enzyme on nucleic acid partially degraded by alkali and the action of alkali on nucleic acid digested by the enzyme. The observations support the suggestion by Loring (4) that ribonucleic acid contains two different types of linkages between mononucleotides, which, according to Gulland *et al.* (6), might arise from nucleotide side chains to the main polynucleotide trunk.

Procedure

Commercial nucleic acids (free acid or sodium salt) were used as obtained or after purification by precipitation with 5 volumes of glacial acetic acid (7). Crystalline ribonucleinase was prepared, and its activity determined by the manometric method, as in previous studies (5, 7).

Precipitation of Nucleic Acid and Mononucleotides by Uranium Chloride in Trichloroacetic Acid—The nucleic acids were characterized by and the course of their hydrolysis followed by their precipitation with uranium

chloride in trichloroacetic acid. According to MacFadyen (8), nucleic acid is completely precipitated by this reagent at pH 1.5 to 2.0, whereas the least soluble mononucleotides are precipitated only in the pH range of 3.0 to 7.0. In the beginning of the present studies this reagent was made up in the proportions used by Kunitz (1): 0.25 per cent uranium chloride in 2.5 per cent trichloroacetic acid. 1 per cent solutions of nucleic acid were used for precipitation; even when strongly alkaline solutions were precipitated a final pH of 1.4 to 1.6 was obtained.[1] This procedure was satisfactory when only small amounts of mononucleotides were present. In the course of alkaline hydrolysis of nucleic acid, however, it was found that a constant amount of precipitate was obtained with the uranium chloride reagent which persisted during prolonged treatment with alkali. The precipitation of mononucleotides was suspected and this was confirmed in experiments of which the results in Table I are typical.

These data show that the mononucleotides adenylic and guanylic acids are precipitated below pH 3.0 and that the pH range of 1.0 to 2.0 is critical; guanylic acid remains in solution at pH 1.00 but would be completely precipitated at pH 1.65; adenylic acid remains in solution at pH 1.50 but is almost completely precipitated at pH 2.00. Essentially the same results were found with both Reagents 1 and 2. It appears also that the two acids precipitate independently (the precipitate from the 0.25 per cent mixture at pH 1.50, for example, is largely accounted for by the guanylic acid expected to be precipitated at that pH) and that at the pH attained in our previous experiments (pH 1.4 to 1.6) only guanylic acid would have precipitated. It is of interest that in a complete hydrolysate of ribonucleic acid with alkali the residue that continued to precipitate with Reagent 1 is about 25 per cent of the nucleic acid, presumably the guanylic acid. In the hydrolytic studies to be reported Reagent 1 was modified by increasing the trichloroacetic acid to 5 per cent and, as before, an equal volume was added to a 1 per cent concentration of nucleic acid or its hydrolysate. Under these conditions the alkaline hydrolysate mentioned above gave a pH of 1.00 and no precipitate.

Hydrolysis of Ribonucleic Acid by Ribonucleinase—The extent of the action of ribonucleinase on several samples of ribonucleic acid is shown in Table II. The maximum hydrolysis (24 hours) was 23 to 33 per cent, almost all of which takes place in the first 2 hours. No further hydrolysis was obtained when additional enzyme was added to Sample E_1, purified, at 20 hours. After hydrolysis the precipitate obtained with an equal volume of 0.5 N HCl is negligible (about 10 per cent of the uranium precipitate), but by adding alcohol as well, the amount of precipitate obtained is proportional

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[1] The values throughout were measured with a Beckman glass electrode pH meter, laboratory model G, calibrated with a standard buffer.

to the alcohol added (1 to 3 volumes). For fractionation of the hydrolysates an amount of alcohol was chosen which would give a weight of precipitate equivalent to the uranium precipitate; this separated the high polymer fraction from the mononucleotides, as confirmed by subsequent test with the uranium reagent. This high polymer fraction was tested with ribonucleinase but no further hydrolysis occurred, measured by the amount

TABLE I

Precipitation of Mononucleotides (Adenylic and Guanylic Acids) by Uranium Chloride in Trichloroacetic Acid, As Function of pH

Mononucleotide concentration*	Reagent No.*†	Solution used to adjust pH	pH	Weight of ppt.‡	Pptd §
				gm.	*per cent*
0.25% each, adenylic and guanylic acids	1	0.5 cc. 10% trichloroacetic acid	1.10	0	0
" "	1		1.25	0.0030	15.0
" "	1	0.2 cc. N NaOH	1.50	0.0120	60.0
0.25% adenylic acid	1		1.28	0	0
0.25% " "	1	0.2 cc. N NaOH	1.50	0	0
0.25% " "	1	0.4 " " "	2.05	0.0120	120.0
0.25% guanylic "	1		1.25	0.0010	10.0
0.25% " "	1	0.2 cc. N NaOH	1.50	0 0090	90.0
0.25% each, adenylic and guanylic acids	2		1.05	0	0
" "	2	0.5 cc. N NaOH	1.45	0 0160	80.0

* The volume of solution and of reagent was 4.0 cc. in each case.

† Reagent 1, 0.25 per cent uranium chloride in 2.5 per cent trichloroacetic acid (1); pH 0.95. Reagent 2, 0.50 per cent uranium chloride in 4.0 per cent trichloroacetic acid (8); pH 0.80.

‡ The precipitates after standing 15 minutes at 25° were sedimented in a centrifuge in tared tubes. The precipitate was spread in a thin layer over the inside of the tube and dried at 70° for 16 hours.

§ Based on the weight of the precipitate and the weight of mononucleotide. The uranium precipitate (Reagent 1) of nucleic acid is about 30 per cent heavier than the nucleic acid and probably the same would be true of the precipitate of the nucleotide. The maximum precipitable in this case would be expected to be 130 per cent.

of the uranium precipitate or manometrically. From this it was concluded that the accumulation of mononucleotides did not explain the termination of hydrolysis at the 23 to 33 per cent level.

Hydrolysis of Ribonucleic Acid with Sodium Hydroxide—The nucleic acid was hydrolyzed in the following manner. 5.0 gm. of nucleic acid (equivalent to approximately 0.015 M of mononucleotides) were adjusted to pH 7 in a volume of 84 cc. and 16.0 cc. of N NaOH were added. Under

these conditions the pH was relatively constant since the NaOH was in excess. Immediately after addition of the alkali a 1.0 cc. sample was taken, diluted with 4.0 cc. of H_2O, and 5.0 cc. of uranium reagent added; the precipitate was collected and dried as before and weighed. Samples were taken at intervals thereafter. An experiment performed in this manner with the nucleic acid Sample EA_2 showed (Fig. 1) that hydrolysis was complete at 25° in 18 to 22 hours. Also shown in the same figure are results with another sample of nucleic acid, "pool" (this was a pool of several samples which was purified by precipitation with acetic acid). The shape

TABLE II

Hydrolysis of Ribonucleic Acid with Ribonucleinase

Sample*	Reactivity measured manometrically†	Weight of ppt. before hydrolysis‡	Decrease in weight of ppt. by maximum hydrolysis§
	c.mm. per 1 γ per hr.	*gm.*	*per cent*
E_1, purified	24.0	0.065	23.1
EA_2	25.6	0.072	33.1
EA_3, purified	28.1	0.083	32.0
Streptococcus	5.8	0.052	28.9
EA_2, NaOH-treated	11.3	0.034	32.4
EA_3, purified, NaOH-treated	12.9	0.048	29.2

* The samples were obtained from the following sources and treated as indicated: E_1, Eastman, free acid, purified; EA_2, Eimer and Amend, sodium salt; EA_3 Eimer and Amend, free acid, purified; streptococcus, isolated from hemolytic streptococcus (9); EA_2, NaOH-treated and EA_3, purified, NaOH-treated; these preparations were partially hydrolyzed with NaOH, as described in the text, Sample EA_2 for 0.5 hour, Sample EA_3, purified, for 2.0 hours. Additional data for these last two preparations are given in Table III.

† 185 mg. of nucleic acid and 30 γ of ribonucleinase in a volume of 3.5 cc. at pH 7.5 and 37° (5, 7).

‡ Weight of precipitate from 5.0 cc. of 1 per cent solution and 5.0 cc. of uranium reagent at 25°.

§ 1 per cent solutions of nucleic acid were hydrolyzed at pH 7.5 and 25° for 24 hours with the ratio of enzyme to substrate 1:1000.

of this curve will be discussed later. Shown in the same figure is the course of hydrolysis of the fraction of Sample EA_2 recovered, after maximum hydrolysis with ribonucleinase, by precipitation with HCl and alcohol, as described earlier. It is clear that this residue is more slowly hydrolyzed than the original nucleic acid.

Similar experiments performed with the nucleic acid, Sample EA_3, purified, are recorded in Fig. 2. Hydrolysis of this nucleic acid is much slower than of Sample EA_2 and it is not significantly altered by the presence of 0.3 M NaCl; nor was it changed by reprecipitation with HCl and alcohol

(same as the procedure used to recover the ribonucleinase-hydrolyzed nucleic acid). Sample EA₃, unpurified, of nucleic acid is more slowly hydrolyzed than Sample EA₃, purified, suggesting that if a contaminating substance is slowing the hydrolysis it may be reduced in amount by precipitation with acetic acid. The fraction of Sample EA₃, purified, of nucleic acid recovered after hydrolysis with ribonucleinase is more slowly hydrolyzed than the parent nucleic acid, in this respect being similar to Sample EA₂.
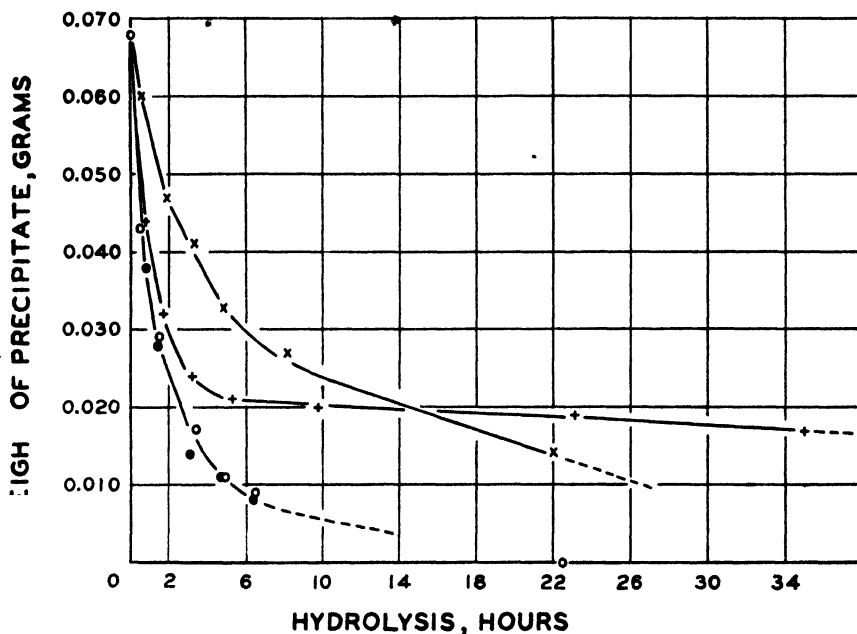
FIG. 1. The course of hydrolysis of ribonucleic acid by sodium hydroxide measured by the precipitate obtained with the uranium reagent. The weights of the precipitates are adjusted to the same initial weight of precipitate as Sample EA₂ . O, ●, nucleic acid Sample EA₂; +, nucleic acid "pool"; ×, nucleic acid fraction recovered from Sample EA₂ after hydrolysis with ribonucleinase.

Hydrolysis by Ribonucleinase of Nucleic Acid Partially Degraded by Sodium Hydroxide—With the data in Figs. 1 and 2 as guide partial hydrolysates of the nucleic acids, Samples EA₂ and EA₃, purified, were prepared, neutralized, and tested manometrically with ribonucleinase. Pertinent data given in Table III show that, although the extent of hydrolysis performed with sodium hydroxide equals (Sample EA₃, purified) or exceeds (Sample EA₂) the maximum obtained with ribonucleinase, the residue is still quite reactive with this enzyme. Furthermore, the data in Table II show that these degraded nucleic acids are hydrolyzed by ribonucleinase to the same degree as the original nucleic acids.
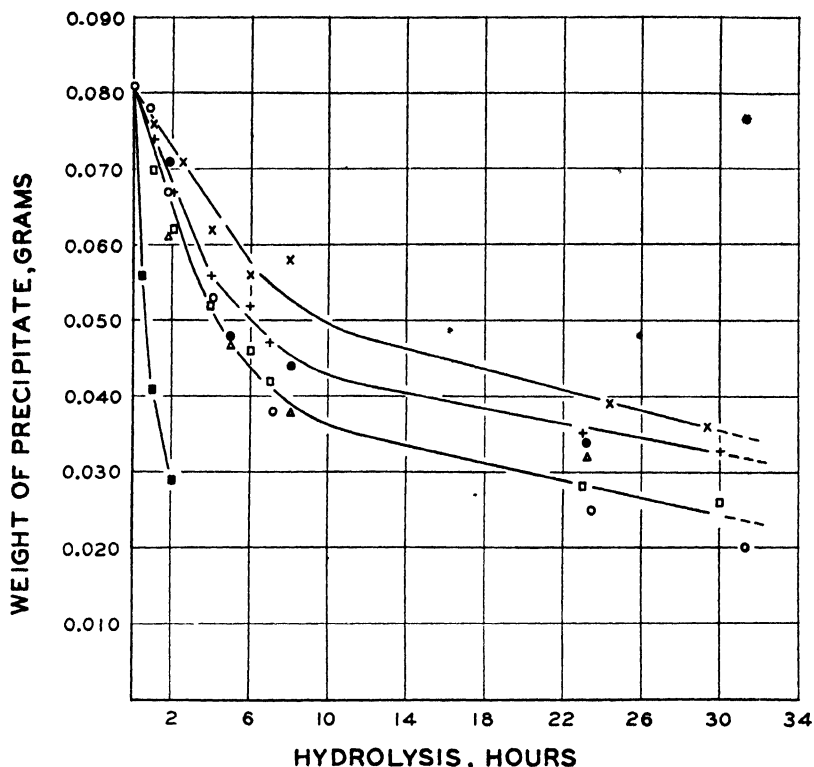
FIG. 2. The course of hydrolysis of ribonucleic acid by sodium hydroxide measured by the precipitate obtained with the uranium reagent. The weights of the precipitates are adjusted to the same initial weight of precipitate as Sample EA₃ , purified. +, nucleic acid EA₃ , unpurified; O, ●, nucleic acid, Sample EA₃ , purified; △, nucleic acid, Sample EA₃ , purified, with 0.3 M NaCl present; □, nucleic acid, Sample EA₃ , purified, after precipitation with HCl and alcohol; ×, nucleic acid fraction recovered from Sample EA₃ , purified, after hydrolysis with ribonucleinase; ■, nucleic acid Sample EA₂ . See Fig. 1 for full curve.

TABLE III

Action of Ribonucleinase on Nucleic Acid Partially Hydrolyzed by Sodium Hydroxide

Sample	Hydrolysis with sodium hydroxide	Unhydrolyzed*	Relative reactivity with ribonucleinase†	Ratio of per cent unhydrolyzed to per cent reactivity
	hrs.	*per cent*	*per cent*	
EA₂	0.5	54	44	1.23
	1.0	37	24	1.54
EA₃, purified	2.0	72	46	1.57
	3.8	67	31	2.16

* Measured by the amount of precipitate obtained with the uranium reagent.

† Calculated as per cent of the reactivity of the original nucleic acid (100 mg.). The NaCl resulting from neutralization of the NaOH has no effect on the activity of the ribonucleinase.

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DISCUSSION

The experiments with uranium chloride in trichloroacetic acid indicate that this reagent should be used at or below pH 1.00 to prevent the precipitation of mononucleotides; at pH 1.05 to 1.65 the principal mononucleotide precipitating would be guanylic acid. Use of this reagent in the past (1, 8) has been in most cases under conditions that would meet the above pH requirement. In some cases (5, 7) the soluble nucleic acid fraction (mononucleotides) was probably low by the amount of guanylic acid precipitated.

The hydrolysis curve of the "pool" nucleic acid (Fig. 1) appears to indicate the presence in the hydrolysate of a fraction other than nucleic acid that is precipitable by the uranium reagent; the pH of the hydrolysate and the pH when mixed with the uranium reagent were satisfactory. The presence of protein was suspected but tests for it were negative. Some contaminating material may cause the mononucleotides to precipitate. The streptococcus nucleic acid (Table II) was known to contain protein (9); after hydrolysis with alkali it gave a precipitate with the uranium reagent of 0.009 gm., which might be protein. The correction by this amount of the hydrolysis data for this nucleic acid in Table II would only change the per cent hydrolyzed from 28.9 to 34.9.

The hydrolysis of Sample EA_3, purified (Fig. 2), was much slower than of Sample EA_2, although performed under the same conditions. These two nucleic acids were about equally reactive with ribonucleinase when tested manometrically (Table II); hence the difference was not due to a difference in polymer size; nor could the difference be due to a difference in the composition of the precipitates, since the reactivity of the residue with ribonucleinase was what one might predict from the hydrolysis measured; nor did the difference in behavior reside in a difference in salt content as shown by the addition of NaCl to Sample EA_3, purified. It should be pointed out that solutions of Sample EA_3 were very much darker than of Sample EA_2. Since Sample EA_3, unpurified, was more slowly hydrolyzed than Sample EA_3, purified, precipitation with acetic acid may have eliminated some material slowing the hydrolysis.

Previous experiments (10) had shown that treatment of ribonucleic acid with H_2O_2 greatly reduced the color present but did not change the reactivity with enzyme nor precipitability with acid. The nucleic acid Sample EA_3 was treated in this manner with the thought that, if the dark colored substances were affecting the rate of hydrolysis, change of their character by oxidation might cause a change in the rate of hydrolysis. The hydrolysis curve of the treated product, however, was identical with that for Sample EA_3, purified, shown in Fig. 2. This interesting difference in rate of hydrolysis with alkali which has been observed before (11, 12) apparently does not affect the hydrolysis by ribonucleinase (Table II).

The precipitation of Sample EA_3, purified, with HCl and alcohol did not improve its hydrolysis. This latter experiment was also useful in interpreting the hydrolysis by alkali of the ribonucleinase-hydrolyzed nucleic acid, since this was the procedure used in its recovery. Both Samples EA_2 and EA_3, purified, after ribonucleinase hydrolysis were more slowly hydrolyzed by sodium hydroxide. The interpretation of this finding is not apparent but the data support the hypothesis that two different types of bonds between mononucleotides exist in ribonucleic acid. The strongest evidence for this conclusion, however, was given by the data for the action of ribonucleinase on nucleic acid partially degraded by sodium hydroxide (Table III). If the linkages between mononucleotides were all alike, some factor like the size of the nucleic acid molecules would necessarily have to be critical in limiting the extent of enzyme hydrolysis. The experiments in Table III show that this cannot be true, for the hydrolysis performed with sodium hydroxide exceeded the maximum produced by the enzyme and yet these degraded nucleic acids were still acted upon by ribonucleinase. Further, these alkali-degraded nucleic acids were still hydrolyzed by ribonucleinase to the same extent (Table II) as the highly polymerized nucleic acids. The increase in the ratio of per cent unhydrolyzed to per cent reactivity probably reflects the accumulation of the inhibiting mononucleotides (5) and the less reactive tetranucleotides.

SUMMARY

The course of hydrolysis of ribonucleic acid was measured by precipitation of the unhydrolyzed nucleic acid with uranium chloride in trichloroacetic acid; the optimum pH for the use of this reagent was shown to lie at or below pH 1.00. The incomplete hydrolysis of ribonucleic acid by ribonucleinase was confirmed and hydrolysis was found not to be increased by the removal of the hydrolytic products, the mononucleotides. A streptococcus ribonucleic acid was incompletely hydrolyzed to the same degree as the yeast ribonucleic acid with which these observations were made. Hydrolysis of ribonucleic acid by sodium hydroxide (0.16 N) at 25° was complete in 18 to 22 hours under favorable conditions; some samples of nucleic acid were not hydrolyzed in this time. Ribonucleic acid that had been hydrolyzed with sodium hydroxide to an extent that exceeded the maximum obtained with ribonucleinase was still quite reactive with the enzyme: Ribonucleic acid subjected to maximum hydrolysis with ribonucleinase was more slowly hydrolyzed with sodium hydroxide than the parent nucleic acid.

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THE MICROBIOLOGICAL DETERMINATION OF CERTAIN FREE AMINO ACIDS IN HUMAN AND DOG PLASMA

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In a previous paper (1) we have reported on the application of microbiological amino acid assay procedures to the determination of free leucine, isoleucine, valine, and threonine in dog plasma. Dunn *et al.* (2), using a microbiological method, have determined the free tryptophane content of human plasma. We have further extended these methods to the determination of free arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tryptophane, tyrosine, and valine in plasma samples from normal human subjects. In addition, the amounts of these amino acids present in dog plasma, not previously reported, are also presented.

EXPERIMENTAL

Methods

The human subjects were volunteer freshman medical and dental students who fasted for 18 hours previous to removal of the 50 cc. blood sample used for the test. Clotting was prevented by the use of heparin. The dogs used were mongrels of both sexes fasted 24 hours previous to sampling.

TABLE I

Recovery of Arginine, Histidine, Lysine, Phenylalanine, and Tyrosine Added to Plasma Previous to Precipitation of Protein

Arginine			Histidine			Lysine			Phenylalanine			Tyrosine		
Found per cc. plasma	Added per cc. plasma	Recovery	Found per cc plasma	Added per cc. plasma	Recovery	Found per cc. plasma	Added per cc. plasma	Recovery	Found per cc. plasma	Added per cc. plasma	Recovery	Found per cc. plasma	Added per cc. plasma	Recovery
γ	γ	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*	γ	γ	*per cent*
25.8	35.0	101	13.2	8.6	85	15.8	10.0	98	9.0	37.5	84	7.5	8.0	121
23.1	18.0	92	11.1	8.6	100	29.0	9.0	120	7.5	9.0	104	7.2	8.6	118
37.5	28.6	121	10.5	7.5	115	24.0	14.3	89	11.9	10.7	113	13.8	8.6	100
27.0	21.4	105	9.3	8.6	104	31.5	14.3	72	14.3	14.3	93	10.2	7.5	100
28.8	18.8	92	11.7	8.6	91	19.5	12.1	116	12.3	9.4	109	8.7	8.6	89
Mean.........		102			99			99			101			106
S.D.........		12.4			10.4			19.3			5.8			7.8

TABLE II

Free Amino Acid Content of Plasma from Normal Human Subjects

Reported as micrograms of amino acid per cc. of plasma.

Subject No.	Age	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tryptophane	Tyrosine	Valine	Total of 10 amino acids
	yrs.											
1	37	26.7	11.7	20.7	25.7	30.0	12.5		10.5	17.1	36.0	
2	19	19.8	13.5	16.0	18.5	28.4	11.4	31.5	10.2	12.0	26.7	188.0
3	26	22.2	11.4	14.3	16.6	23.9	11.1		9.6	11.4	32.7	
4	20	18.3	11.1	15.2	19.4	27.0	10.7	18.8	8.7	11.7	26.3	167.2
5	17	15.6	11.5	15.0	16.6	20.0	9.3		10.2	12.9	28.5	
6	19	30.3	12.0	16.8	18.6	36.8	14.6	22.4	12.9	15.3	27.3	207.0
7	18	19.2	14.4	13.7	20.9	24.0	13.7		9.9	12.0	25.7	
8	30	14.1	11.4	13.2	18.9	22.4	11.6	12.8	9.3	13.2	27.2	154.1
10	20	15.6	13.5	17.3	22.9	25.0	10.7	13.4	9.3	12.6	28.2	168.5
11	20	17.4	12.6	19.5	20.4	23.0	10.7		9.9	11.7	26.7	
12	24	14.4	15.0	12.5	16.0	28.4	10.5	18.6	15.9		22.5	
13	20	26.4	15.3	14.7	20.1	32.6	16.9	22.2	15.6	10.5	30.6	204.9
15	27	20.4	17.1	18.6	23.1	35.8	14.7	16.2	12.9	12.3	29.3	200.4
16	28	33.6	16.5	14.3	19.2	36.0	14.3	19.6	13.2	15.6	29.6	211.9
24	21	32.1	16.5	19.7	19.2	34.0	20.0	22.8	11.1	24.6	30.5	230.5
25	20	28.5	21.0	23.3	27.8	36.2	20.6	23.9	12.9	18.6	33.0	245.8
26	19	20.7	18.0	17.3	20.6	27.5	12.3	24.5	10.2	14.4	27.0	192.5
27	18	22.5	15.3	22.5	22.6	31.7	14.7	23.7	12.0	19.8	28.9	213.6
28	21	36.9	13.5	22.5	20.0	36.9	17.1	27.0	10.5	20.1	37.4	241.9
29	22	32.1	12.9	18.9	17.3	28.2	18.2	20.3	14.1	20.7	27.9	210.6
31	19	21.6	15.3	12.3	21.0	32.3	18.5	22.2	6.9	15.9	28.8	194.8
32	20	20.7	13.5	13.8	16.5	30.3	12.6	21.3	7.5	15.0	28.5	179.7
33	19	24.6	14.7	14.9	18.5	28.8	16.7	20.6	12.0	15.6	28.5	194.9
34	26	23.1	13.8	12.8	26.4	33.0	14.7	12.9	12.0	12.0	24.8	185.5
Mean		23.2	14.2	16.6	20.3	29.7	14.0	20.8	11.1	15.0	28.9	199.5
S.D.		6.4	2.4	3.4	3.6	4.6	3.5	4.9	2.3	4.2	3.7	24.3

Males

Subject No.	Age	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tryptophane	Tyrosine	Valine	Total of 10 amino acids
9	26	21.9	14.4	12.8	14.7	28.2	12.5	15.9	12.3	12.3	25.4	170.4
14	18	21.6	13.8	12.8	14.0	24.8	12.0	21.0	10.8	17.1	24.3	172.2
17	26	23.7	15.0	12.8	15.8	26.7	10.7	19.4	9.3	10.8	26.4	170.8
18	34	21.6	13.5	13.0	15.5	26.1	9.6	24.6	9.9	8.7	26.4	168.9
19	25	13.5	14.1	13.5	17.1	27.8	10.0	11.1	10.8	9.0	23.3	150.2
20	18	22.5	12.3	14.0	12.6	25.8	10.5	15.6	7.8	13.2	25.2	159.5
22	24	24.3	13.5	16.5	18.0	35.1	15.7	23.3	8.4	18.9	32.0	205.7
23	30	32.4	14.1	15.5	18.8	33.8	18.6	18.6	8.7	19.5	25.2	205.2
30	21	33.3	15.3	16.2	16.0	31.5	15.8	20.6	9.9	18.3	32.6	209.5
Mean		23.9	14.0	14.1	15.8	28.9	12.8	18.9	9.8	14.2	26.7	179.1
S.D.		6.0	0.8	1.7	2.1	3.2	3.2	3.9	1.1	4.3	3.6	21.1

Females

<div align="center">Table II—Concluded</div>

<div align="center">Recapitulation for all subjects</div>

Mean..	23.4	14.2	16.0	19.1	29.5	13.8	20.2	10.8	14.8	28.3	192.8
s.d..	6.2	1.8	3.1	3.4	4.2	3.2	4.5	2.1	3.7	3.4	24.8

The preparation of the plasma and of the tungstic acid filtrates was carried out as previously described (1). The basal media and organisms for the assay of arginine, histidine, isoleucine, leucine, lysine, phenylalanine, threonine, tyrosine, and valine were the same as described by Hier *et al.* (3). Only 1.0 cc. of basal medium was added to each tube. After the addition of standard and unknown solutions the final volume was adjusted to 2.0 cc. In other details the procedure previously described (1, 3) was followed. Tryptophane values were obtained by use of the procedure of Dunn *et al.* (2).

Recovery and Reproducibility Experiments—The quantitative nature of the procedures applied to the determination of leucine, isoleucine, valine, and threonine in plasma has previously been shown (1) by means of recovery and reproducibility experiments. Similar tests were applied to the determination of arginine, histidine, lysine, phenylalanine, and tyrosine. The amino acids to be recovered were added in solution to the plasma previous to precipitation with sodium tungstate and the amount of water added was reduced accordingly to maintain a 1:3 ratio between the plasma and filtrate. Table I shows that recoveries were satisfactory for all of the amino acids studied.

Duplicate assays made on the same tungstic acid filtrate samples indicated that values were reproducible to within ±10 per cent of the mean.

Amino Acid Content of Human Plasma—The results of assays for ten free amino acids in plasma from twenty-four normal males and nine normal females are shown in Table II. It may be seen by observation and demonstrated statistically that there is no significant difference between the mean values for males and females. Our value of 11.1 γ per cc. for tryptophane in male subjects agrees extremely well with the value of 1.14 mg. per cent (11.4 γ per cc.) reported by Dunn *et al.* (2).

Amino Acid Content of Dog Plasma—The free arginine, histidine, lysine, phenylalanine, tryptophane, and tyrosine contents of plasma from mongrel dogs are shown in Table III. Leucine, isoleucine, valine, and threonine values have previously been reported (1).

<div align="center">DISCUSSION</div>

The average amounts of arginine, histidine, lysine, phenylalanine, tyrosine, and tryptophane found in dog plasma (Table III) are substantially the same as the average values for human subjects (Table II). The values

for leucine, isoleucine, valine, and threonine in dog plasma previously reported (1) (20.9, 13.1, 22.2, and 25.8 γ per cc. respectively) are also quite close to the values reported in this paper for human subjects (Table II).

In an effort to determine whether the individual free amino acids vary with the total amount of free amino acids, each amino acid was calculated in terms of per cent of the total amount of the ten amino acids determined in plasma. These values are shown in Table IV. The size of the standard deviations in relation to the mean indicates that, while the range of values is fairly characteristic for each amino acid, the level of any one amino acid may vary within the normal range from subject to subject independently of the total amount of amino acids. Isoleucine, histidine, and phenylala-

TABLE III

Free Amino Acid Content of Plasma from Fasted Normal Dogs

Reported as micrograms of amino acid per cc. of plasma.

Dog No.	Arginine	Histidine	Lysine	Phenylalanine	Tryptophane	Tyrosine
1	25.8		15.8	9.0		7.5
2	23.1		29.0	7.5		
3		13.2	24.0	11.9		7.2
4	37.5	11.1	31.5	14.3		13.8
5		10.5	19.5	12.3		10.2
6	27.0	9.3	24.0	10.0	9.1	4.5
7	28.8	11.7	15.0	18.0	7.5	8.7
8	48.0	12.9	35.0	15.2	9.6	19.8
9	27.0	11.4	17.6	12.5	13.2	15.3
10	37.2	13.5	26.6	12.5	12.9	11.1
11	36.0	15.9	24.2	7.0	10.2	7.8
12	36.9	14.4	28.0	8.6	10.2	10.2
Mean	32.7	12.4	24.2	11.6	10.4	10.6
S.D.	7.4	1.7	5.8	2.9	1.7	2.4

nine may be exceptions to this, since they appear to be more closely related to the total.

In Table V a comparison is made between the mean values for the per cent that each amino acid makes up of the total amount of the ten amino acids determined in plasma (from Table IV), with similar figures calculated from the amounts of the ten amino acids present in various animal proteins. With the exception of tryptophane,[1] the original values for beef muscle

[1] The tryptophane values are calculated from unpublished data, kindly supplied by Dr. C. E. Graham of The Wilson Laboratories, determined by a modification of the Bates method (Bates, R. W., *Proc. Am. Soc. Biol. Chem., J. Biol. Chem.*, **119**, p. vii (1937)).

protein, bovine plasma protein, bovine serum albumin, bovine fibrin, and casein were reported by Hier *et al.* (3). The data for porcine liver protein and bovine hemoglobin were obtained by similar methods but are unpublished.

TABLE IV

Concentration of Each Free Amino Acid Expressed as Per Cent of Total Ten Amino Acids Determined

Subject No.	Arginine	Histidine	Isoleucine	Leucine	Lysine	Phenylalanine	Threonine	Tryptophane	Tyrosine	Valine
2	10.5	7.2	8.5	9.8	15.1	6.1	16.8	5.5	6.4	14.2
4	11.0	6.6	9.1	11.6	16.2	6.4	11.3	5.2	7.0	15.8
6	14.7	5.8	8.2	9.0	17.8	7.2	10.9	6.3	7.4	13.2
8	9.1	7.4	8.6	12.2	14.5	7.5	8.3	6.0	8.6	17.6
9	12.8	8.4	7.5	8.6	16.5	7.3	9.3	7.2	7.2	14.9
10	9.2	8.0	10.2	13.6	14.8	6.3	7.9	5.5	7.5	16.7
13	12.9	7.5	7.2	9.8	15.8	8.3	10.8	7.6	5.1	15.0
14	12.5	8.0	7.8	8.1	14.4	7.0	12.2	6.3	9.9	14.1
15	10.2	8.5	9.3	11.6	17.9	7.4	8.1	6.5	6.2	14.7
16	15.8	7.8	6.7	9.1	17.0	6.8	9.3	6.4	7.4	14.0
17	13.9	8.8	7.5	9.2	15.6	6.4	11.3	5.4	6.3	15.4
18	12.8	8.0	7.7	9.2	15.5	5.7	14.6	5.9	4.8	15.6
19	8.9	9.3	8.9	11.3	18.3	6.6	7.4	7.2	6.0	15.4
20	14.1	7.7	8.8	7.9	16.2	6.6	9.8	4.9	8.3	15.8
22	11.8	6.6	8.3	8.8	17.1	7.6	11.3	4.1	9.2	15.6
23	15.3	6.9	7.5	9.2	16.5	9.1	9.1	4.2	9.5	12.3
24	13.9	7.2	8.5	8.3	14.7	8.7	9.9	4.8	10.7	13.2
25	11.3	8.6	9.5	11.3	14.7	8.3	9.8	5.3	7.5	13.4
26	10.7	9.2	9.0	10.8	14.3	6.4	12.7	5.3	7.5	14.0
27	10.5	7.2	10.5	10.5	14.8	6.9	11.1	5.6	9.3	13.5
28	14.8	5.6	9.3	8.3	15.3	7.1	11.2	4.4	8.3	15.5
29	15.2	6.1	8.9	8.1	12.9	8.6	9.6	6.7	9.8	13.2
30	15.9	7.3	7.8	7.7	15.0	7.6	10.0	4.7	8.7	15.6
31	11.1	7.9	6.3	10.8	16.6	9.5	11.8	3.5	8.2	14.8
32	11.5	7.5	7.7	9.2	16.9	7.0	11.8	4.2	8.4	15.8
33	12.6	7.5	7.6	9.5	14.8	8.6	10.6	6.1	8.0	15.2
34	12.5	7.4	6.9	14.2	17.8	7.9	6.9	6.5	6.5	13.4
Mean.	12.4	7.6	8.3	9.9	15.8	7.4	10.5	5.6	7.8	14.7
s.d..	2.13	0.40	0.58	1.68	2.43	0.66	2.14	1.15	1.25	2.74

It may be seen from Table V that there is rather good agreement between the pattern of amino acids in beef muscle protein and that of free amino acids in plasma. The only large differences occur in the case of tryptophane and leucine. The pattern in plasma proteins is also similar, while that of liver protein, serum albumin, fibrin, hemoglobin, and casein shows con-

siderable variance. This may indicate a relationship between the free amino acids of the plasma and the protein of plasma and muscle. A possible relationship to particular protein fractions of other tissues is, of course, not excluded.

Although we do not present data for human proteins, we believe it reasonable to compare the pattern of free amino acids in human plasma with the amino acid pattern of bovine and porcine proteins and to assume that the same relationships hold true for human proteins. Beach *et al.* (4) have shown that mammalian muscle tissues of various species are very similar in amino acid composition, while Block and Bolling (5) report amino acid

TABLE V

Comparison of Pattern of Free Amino Acids in Plasma with Pattern of Amino Acids in Various Animal Proteins

Each amino acid is calculated as the per cent of the total of the ten amino acids determined.

Amino acid	Free amino acids in human plasma	Bovine muscle protein	Bovine plasma protein	Porcine liver protein	Bovine serum albumin	Bovine fibrin	Bovine hemoglobin	Casein
Arginine	12.4	11.4	9.6	13 4	9.1	12.7	6.8	6.7
Histidine	7.6	6.7	4.7	4 1	6.1	3.9	9.5	4 9
Isoleucine	8.3	10.2	6.1	12.4	4.9	10.4	1.9	11.2
Leucine	9.9	14.6	15.8	19.2	16.8	12.2	24.6	17.7
Lysine	15.8	15 0	19.0	11 4	18 3	15.0	17.3	14.2
Phenylalanine	7 4	7.5	8.5	7.4	9.5	7.8	11.8	7 9
Threonine	10.5	10.3	11.8	11 2	9 4	11.3	8.1	7.7
Tryptophane	5 6	2.1	3.5	1 8	1 6	6.4	2.5	2.2
Tyrosine	7.8	7 8	8.2	6 9	7 7	10.6	4.1	12.2
Valine	14 7	14.7	13.0	11.8	16 8	9.7	13.7	13.3

values indicating the close similarity between human and beef serum proteins.

SUMMARY

Microbiological procedures for the determination of free amino acids in plasma are described and evidence for the reproducibility and validity of the values is presented. Values are reported for the amounts of arginine (23.4 ± 6.2 γ per cc.), histidine (14.2 ± 1.8 γ per cc.), isoleucine (16.0 ± 3.1 γ per cc.), leucine (19.1 ± 3.4 γ per cc.), lysine (29.5 ± 4.2 γ per cc.), phenylalanine (13.8 ± 3.2 γ per cc.), threonine (20.2 ± 4.5 γ per cc.), tryptophane (10.8 ± 2.1 γ per cc.), tyrosine (14.8 ± 3.7 γ per cc.), and valine (28.3 ± 3.4 γ per cc.) present in the free state in normal human plasma. No significant differences between values for males and females

were found.   The amounts of free arginine, histidine, lysine, phenylalanine, tryptophane, and tyrosine in dog plasma are also reported.

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l-HYDROXY ACID OXIDASE

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In previous communications (1–4) the properties and isolation of the
l-amino acid oxidase of rat kidney were described. Quite accidentally, it
was found that solutions of the enzyme were able to catalyze the oxidation
of l-hydroxy acids, and further study disclosed that this ability ran parallel
with l-amino acid oxidase activity from the first crude extract to the final
electrophoretically homogeneous preparation of the enzyme. The experi-
ments which are reported below support the view that the same enzyme
exercises these two catalytic functions and that the same prosthetic group
and active groups are involved in both cases. In a sense, the enzyme is
incapable of distinguishing between an amino acid and a hydroxy acid of the
l series.

There are present in practically all animal tissues enzymes such as the
lactic and malic dehydrogenases which specifically oxidize certain l-hydroxy
acids. These enzymes can be distinguished from the l-hydroxy acid
oxidase in two respects: they are specific for only one hydroxy acid and they
specifically require diphosphopyridine nucleotide as oxidizing agent (5).
By contrast, the enzyme isolated from rat kidney oxidizes a large variety of
α-hydroxy acids, does not require or react with diphosphopyridine nucleo-
tide, and contains flavin monophosphate as prosthetic group. It is clear
that the enzyme is distinct from other hydroxy acid oxidizing systems.
Thus far hydroxy acid oxidase activity has been found only in conjunction
with amino acid oxidase activity. The structural analogy between the
α-amino and α-hydroxy acids makes it appear very probable that the
mechanism of oxidation, involving the amino or hydroxyl hydrogen as well
as the hydrogen attached to the α-carbon atom, is the same in both classes
of substrates. This is borne out by the fact that α-hydroxyisobutyric acid,
which has no hydrogen on the α-carbon atom, is not oxidized.

It was of interest to determine whether the d-amino acid oxidase of
animal tissues was also active towards the d isomers of the α-hydroxy acids.
Using the enzyme from pig kidney, we have tested dl-lactate and dl-
hydroxy caproate, with negative results. Apparently the ability to
oxidize hydroxy acids is not a general property of amino acid oxidases.

It has not yet been possible to demonstrate the reversibility of the α-hydroxy or α-amino acid functions of the *l*-amino acid oxidase. If the action of the *l*-amino acid oxidase were reversible, it would be possible to account for the transformation *in vivo* of certain natural amino acids into hydroxy acids, as reported in the older literature (6). This transformation could be explained simply in terms of the oxidation of amino acids to keto acids and the reduction of these keto acids to their corresponding hydroxy

FIG. 1 The rate of oxidation of lactate as a function of time. The following additions were made in the manometer vessels: 0 5 cc. of enzyme solution, 0.2 cc. of 50 per cent *dl*-lactate, 0.5 cc. of catalase solution, 1 0 cc. of 0.5 M phosphate buffer of pH 8.0, final volume 3 0 cc. at 38°; air in gas space; alkali in center well.

acids. The observation of Neubauer that the feeding of phenylglyoxylic acid leads to the excretion of the natural isomer of phenylglycolic acid (6) may also be accounted for on the same basis.

Preparation and Testing of the Enzyme—The details of the preparation are identical with those previously reported for the *l*-amino acid oxidase. The action of the enzyme can be followed aerobically in Warburg manometers or anaerobically in Thunberg tubes. The experimental conditions for those measurements can be found in previous communications (2, 4).

Kinetics—In presence of lactate and catalase the enzyme takes up oxygen linearly for about 30 minutes and then the rate gradually falls off (*cf*. Fig. 1). The greater the initial rate of the reaction, the more rapidly does the decline in rate set in.

The velocity of oxidation is greatly influenced by the hydrogen ion concentration (*cf*. Fig. 2). The maximum pH is about 8.0, whereas above pH 10 and below pH 6 the rate of oxidation approaches zero. The enzyme works at maximum speed when the substrate concentration is about 0.04 M and at half maximum speed when the concentration is about 0.004 M (*cf*. Fig. 3).
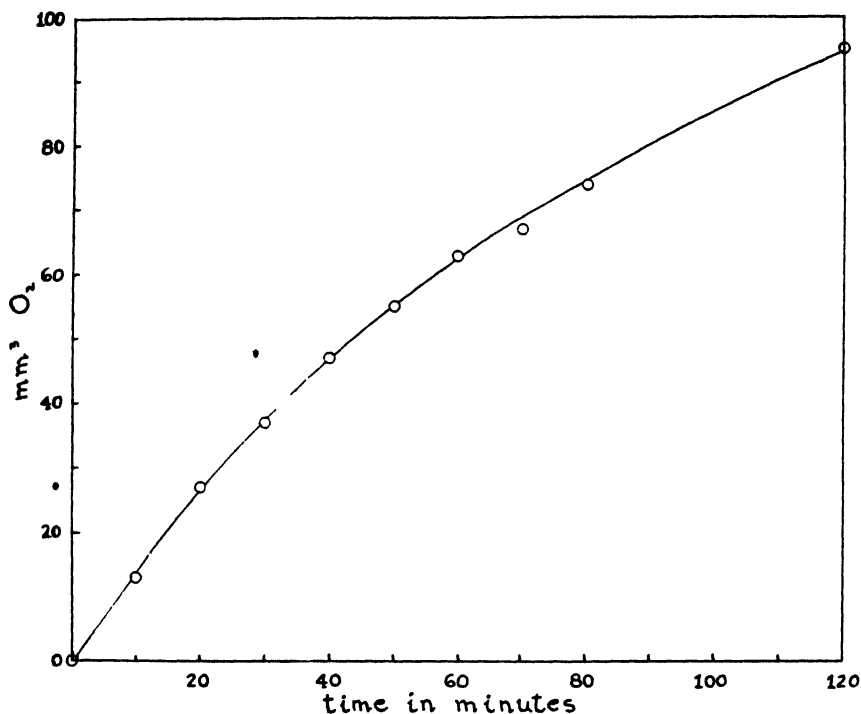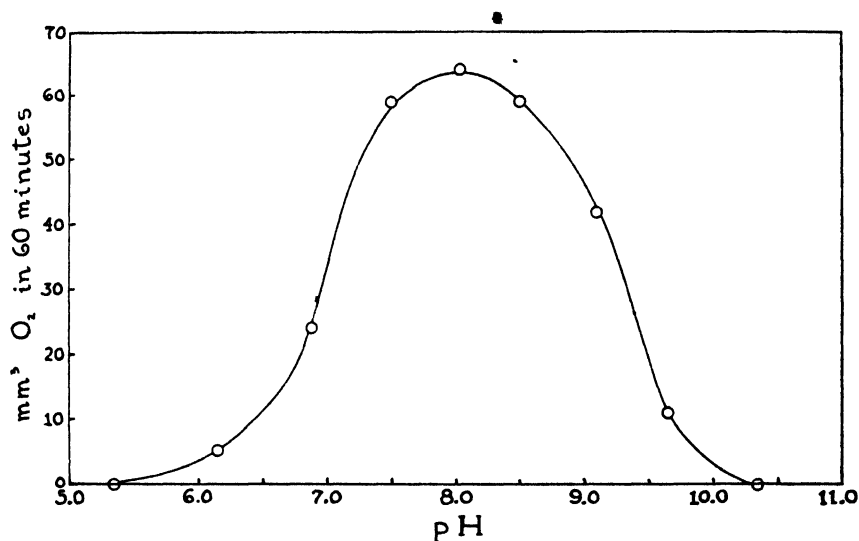

FIG. 2. The rate of oxidation of lactate as a function of the pH. The following additions were made in all manometer vessels: 0.5 cc. of enzyme solution, 0.5 cc. of 5 per cent *dl*-lactate, 0.5 cc. of catalase, and 0.5 M buffer to make a final volume of 3.0 cc. All solutions were adjusted to the pH of the buffer. The pH of the enzyme mixture was measured at the end of the manometric run.

Specificity—The enzyme apparently acts only on the *l* antipodes of α-hydroxy acids. While it has not been shown directly that *d*-hydroxy acids are not oxidized, this conclusion follows from the fact that *l*-lactic acid is completely oxidized, whereas *dl*-lactic acid is oxidized only to the extent of 50 per cent (*cf*. Table I). α-Hydroxyisobutyric acid was the only one of fourteen hydroxy acids tested which was not oxidized at a measureable rate by the enzyme (*cf*. Table II). The presence of a second carboxyl group renders the substrate inactive in the amino acid series and slowly oxidizable in the hydroxy acid series. Thus aspartic acid is inactive as

substrate and malic acid is very slowly oxidized. The fact that glycolic acid is oxidized, albeit slowly, signifies that the enzyme can oxidize an optically inactive substance and is capable of distinguishing sharply between two optical isomers.

In general the specificities of the *l*-amino acid oxidase are similar to those of the *l*-hydroxy acid oxidase. For example, with amino acids as substrates there is a very pronounced relation between the length of the carbon chain and the rate of oxidation. Thus, in the straight chain series,
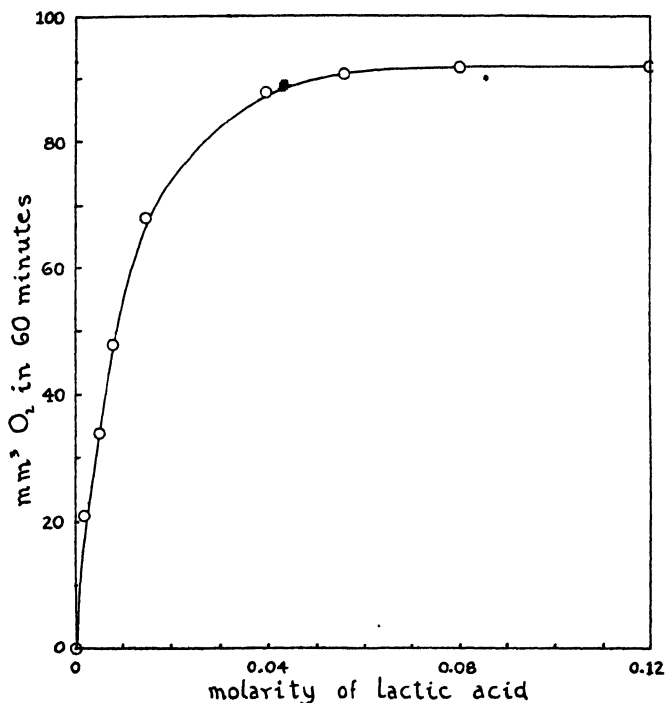
Fig. 3. The rate of oxidation of lactate as a function of the concentration of lactate. Zinc *l*(+)-lactate was used as the source of lactic acid. Conditions as given in the legend for Fig. 2.

the amino acid with 5 carbon atoms is most rapidly oxidized, the next lower homologue is slowly oxidized, whereas those with 3 and 2 carbon atoms respectively are oxidized very slightly or not at all. With hydroxy acids as substrates, the homologues with 4 and 5 carbon atoms are only slightly more rapidly oxidized than lactate with 3 carbon atoms, which in turn is oxidized some 6.5 times more rapidly than glycolic acid with 2 carbon atoms. Arginine and serine are not oxidized by the *l*-amino acid oxidase, whereas histidine is slowly oxidized. The hydroxy acids corresponding to arginine, serine, and histidine are all slowly oxidized.

Products of Reaction—In presence of catalase and the enzyme 1 atom of oxygen is taken up for each mole of hydroxy acid oxidized and 1 mole of keto acid is formed, as shown in Table II. Pyruvate was identified as the product of the oxidation of lactate in the form of its 2,4-dinitrophenyl-hydrazone which was obtained in 79 per cent yield, m.p. (with decomposition) 214°, mixed m.p. 214°.

Analysis—$C_9H_8O_6N_4$. Calculated. C 40.3, H 2.98, N 20.9
Found. " 40.5, " 3.16, " 20.8

Column 2 of Table II shows the observed uncorrected values for the individual keto acids as estimated by iodine titration of the bisulfite-binding power. These values are somewhat low when compared directly with the corresponding values for oxygen uptake. ·There is no doubt, however,

TABLE I

Optical Specificity of Enzyme

Substrate	Oxygen uptake		
	Observed	Theory for oxidation of 1 isomer	Theory for oxidation of both isomers
	c.mm.	*c.mm.*	*c.mm.*
Zinc *l*(+)-lactate.................	100	99	
	197	194	
dl-Lactate*.......................	110	95	190
	221	190	380
dl-Lactate†.......................	73	99	198

* A commercial sample obtained by a fermentation process and presumed to be racemic.

† Prepared by hydrolysis of *dl*-α-bromopropionic acid. The manometric experiments were carried out in air at 38° and at pH 8.0, and in the presence of catalase.

that 1 mole of keto acid is formed for each mole of oxygen utilized; for it has been shown that less than one equivalent of bisulfite is bound by most keto acids other than pyruvic. The method may be employed satisfactorily nevertheless if a previously determined correction factor is applied (2). Column 3 of Table II gives some such corrected values, and these are in good agreement with the amount of oxygen utilized.

In absence of catalase 2 atoms of oxygen should be taken up per mole of lactate oxidized and 1 mole of hydrogen peroxide should be formed. Variable amounts of peroxide are decomposed by non-enzymic means—a factor which makes it impossible to obtain stoichiometric results. Experiments in which hydrogen peroxide is allowed to accumulate are also unsatisfactory because the enzyme undergoes rapid destruction under these conditions. In the presence of alcohol and catalase the rate of oxygen uptake is doubled.

TABLE II

Relation between Oxygen Uptake and Amount of Keto Acid Produced

Substrate	Oxygen uptake (1)	Amount of keto acid formed uncorrected (2)	Amount of keto acid formed corrected (3)
	microatoms	*micromoles*	*micromoles*
dl-α-Hydroxy isovalerate*	38 1	29 5	
dl-α-Hydroxy butyrate*	31 4	29 4	
l-α-Hydroxy isocaproate†	29 8	25 8	29 6
dl-α-Hydroxy caproate*	28 7	25 5	28 0
dl-Phenyl glycolate‡	24 1	12 5	
dl-Lactate*	23 4	22 5	23 0
dl-Phenyl lactate†	17 8	15 2	17 1
dl-α-Hydroxy-γ-methiobutyrate†	17 4	17 3	
l-Imidazolelactate†	8 8		
dl-Glycerate§	8 3		
dl-Malate‡	6 7		
Glycolate‡	5 2	2 7	
dl-α Hydroxy δ guanidovalerate†	1 5	0 4	
α-Hydroxy isobutyrate‡	0	0	

The conditions for the manometric experiments were as follows 1 cc of enzyme solution, 0 5 cc of catalase solution prepared as described previously (3), 1 cc of 0 5 m phosphate buffer of pH 8 0, and 0 5 cc of 0 1 m solution of substrate adjusted to pH 8 0, alkali in center well, 38°, air in gas space, time 100 minutes   The keto acids were estimated as described before (2)

* Prepared from the corresponding *α*-bromo acid, according to Fischer and Zemplen (7)

† Prepared from the corresponding amino acid by treatment with silver nitrate, as described by Schoenheimer *et al* (8)

‡ Commercial samples purchased from the Eastman Kodak Company

§ Prepared from glyceric aldehyde by oxidation with mercuric oxide, according to Wohl and Schellenberg (9)

TABLE III

Association of Amino Acid Oxidase with Hydroxy Acid Oxidase Activity

Stage of purification	Rate of oxidation of lactic acid / Rate of oxidation of leucine
1st sodium sulfate ppt	2.8
After exposure to 60° for 5 min	2 7
2nd sodium sulfate ppt	2 6
Ultracentrifuge fractionation of cataphoretically homogeneous enzyme	
Light fraction	2 0
Middle "	2 2
Heavy "	2 2

The two activities were measured at pH 8 7, 38°, in air and in the presence of added catalase   The final concentration of substrate was 0 01 m for both *l*-lactate and *l*-leucine.   For further details of the method of isolation of *l*-amino acid oxidase see Blanchard *et al* (4).

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This constitutes the most reliable proof that peroxide is formed during the enzymic oxidation of lactate and other hydroxy acids by molecular oxygen (10).

Identity of l-Amino Acid Oxidase and l-Hydroxy Acid Oxidase—If the two enzymes are identical, it would be expected that the ratio of the two activities would be a constant throughout the purification procedure, within the limits of experimental variation. That such is indeed the case is shown in Table III. The electrophoretically homogeneous enzyme consists of two forms of molecular weight of 138,000 and 550,000 respectively. These forms were separated in the ultracentrifuge and examined for the ratio of the two catalytic activities. No significant difference in the ratio was found.

The *l*-amino acid oxidase is a flavoprotein which undergoes a cycle of reduction by *l*-amino acids and oxidation by molecular oxygen. If the two activities are shared by the same enzyme, then the same flavin prosthetic group which is reducible by *l*-amino acid should also be reducible by *l*-hydroxy acids. Spectrophotometric examination has confirmed this prediction. Furthermore, the rate of reduction of the flavin group by hydroxy acids is more rapid than in the presence of the corresponding amino acids in accordance with the fact that the enzyme acts more rapidly on hydroxy than on amino acids.

The third line of evidence for identity is the fact that the two activities do not summate but in fact partially interfere with one another. This behavior is consistent with the view that the two activities are associated with the same enzyme and with the same active groups of the enzyme.

The *l*-amino acid oxidase activity is inhibited by ammonium ions in low concentration, whereas the *l*-hydroxy acid oxidase function is virtually unaffected by ammonium ions. This would suggest that the enzyme has an affinity for ammonium ions at least equal to that for amino acids but much less than that for *l*-hydroxy acids.

SUMMARY

The properties of the *l*-hydroxy acid oxidase of rat kidney are described and evidence is presented for its identification with the *l*-amino acid oxidase.

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THE ACTION OF TYROSINASE ON PROTEINS

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Tyrosinase catalyzes the oxidation by oxygen of a large variety of mono- and polyphenols (1, 2) as well as many phenolic derivatives such as adrenalin, certain sex hormones, tyrosine, and the poison ivy irritants (3, 4). Monophenols are oxidized to diphenols; these are in turn converted to unstable quinone derivatives which polymerize to highly colored compounds of unknown structure. A typical example is the aerobic oxidation of tyrosine by tyrosinase to 3,4-dihydroxyphenylalanine (dopa), which is converted by way of unstable intermediaries to the pigment melanin (5, 6). This oxidation of tyrosine is believed to be involved in the production of pigmentation in many different organisms.

As far as the author is aware the only enzymes which are known to attack simple intact proteins *in vitro* are proteolytic ones, although chemical reagents can readily bring about modifications in such constituents of native proteins as amino, carboxyl, and phenolic groups. The situation *in vivo* appears to be quite different, however, for tracer studies by Schoenheimer (7) and others have clearly shown that proteins in the body are quite labile and that substitutions of amino acids appear to occur and that reactions take place which involve active available groups on the protein molecule. A consideration of the discrepancy between the reactivity *in vivo* and *in vitro* of proteins to non-proteolytic enzymes led to this study of the action of tyrosinase on proteins. In view of the low specificity of tyrosinase it was felt that if the tyrosine residue were available at the surface of the protein molecule its phenolic hydroxyl group might be subject to attack by mushroom tyrosinase.

Methods and Results

Manometric Experiments—The Barcroft differential manometer was used, at 37° ± 0.02° and at a shaking rate of about 120 oscillations per minute, to study any effect of tyrosinase in bringing about an oxygen consumption by the protein solutions. The reaction flasks had a volume of 15 ml. and possessed a single side arm in which the enzyme was placed. The experimental flask contained 25 mg. of protein, 4.5 ml. of M/15 phosphate buffer, pH 7.3, 2 drops of toluene (or 0.001 per cent merthiolate), and 0.5 ml. of tyrosinase solution in the side arm. The control flask contained

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exactly the same components as the experimental, except that boiled enzyme was placed· in the side arm. In some experiments, however, distilled water was used in the control instead of the inactivated tyrosinase. After 5 minutes adaptation to temperature, the reactions were initiated by tipping the enzyme into the protein solution.   In most experiments Upjohn mushroom tyrosinase was used, which contained 1100 Adams and Nelson catechol-hydroquinone units, 150 cresolase units, and 2750 chrono-metric catecholase units per ml. A few experiments with very similar results were also performed with Nelson's highly purified mushroom
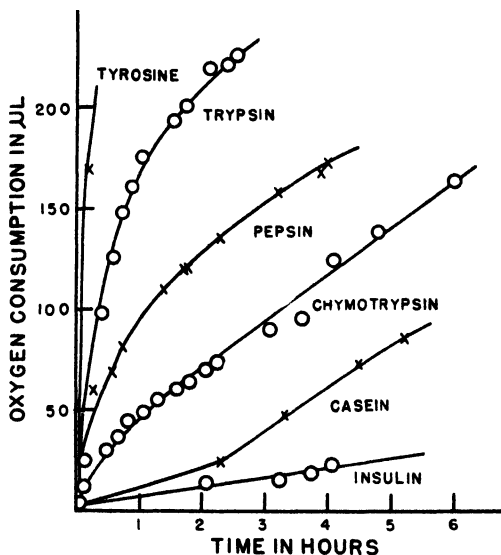
FIG. 1.   Oxygen consumption of proteins in the presence of tyrosinase.   The solu-tion contained 25 mg. of protein, 4.5 ml. of M/15 phosphate buffer, pH 7.3, and 0.5 ml· of Upjohn tyrosinase, plus 2 drops of toluene.   The tyrosine solution contained only 2.5 mg. of tyrosine, 4.95 ml. of buffer, and 0.05 ml. of tyrosinase.

tyrosinase,[1] which contained 4200 Miller and Dawson catecholase units and 70 Adams and Nelson p-cresolase units per ml. (1).

Results of typical experiments are presented in Fig. 1, from which it appears that the action of tyrosinase in the oxidation of certain proteins is clearly demonstrated by the oxygen consumption of protein solutions in-duced by the addition of tyrosinase.   The reaction follows a smooth cur-vilinear course characteristic of the particular protein substrate employed, but the rate is much less than for the action of 0.05 ml. of tyrosinase on 2.5 mg. of l-tyrosine.   Results for all the proteins studied are summarized

[1] Kindly furnished by Dr. J. F. Nelson of Columbia University.

TABLE I

Action of Tyrosinase on Proteins with Reference to Oxygen Consumption, Pigment Production, and Reaction to Millon's Reagent

Substrate		Absorption in blue-violet	Millon's test*
	microliters O_2 per hr.		
l-Tyrosine†	250	+	−
Trypsin‡	163	+	−
Pepsin‡	90	+	−
Chymotrypsin‡	43	+	−
Peptone (Difco)	58	+	−
Casein§	11	+	−
Zinc insulin (Merck)	9	+	−
Hemoglobin (Difco)	2	+	−
Gelatin (Difco)	0	−	−
Protamine (Lilly)	0	−	−
Tobacco mosaic virus‖	0	−	Not measured
Egg albumin (Merck)	0	−	+
Serum albumin, human¶	0	−	+
" " " + 1 mg. trypsin	6	+	−
" " bovine**	0	−	Not measured
" " " + 1 mg. trypsin	0.5	+	−
" γ-globulin, human¶	0	−	+
" " " + 1 mg. trypsin	1	+	−
" β-globulin, bovine**	0	−	Not measured
" " " + 1 mg. trypsin	2	+	−
Fibrinogen, bovine**	0.1	±	Not measured
" " + 1 mg. trypsin	13	+	−
Gramicidin§§	0	−	−
" + 1 mg. trypsin	0	−	−

* If the production of a pink solution or precipitate by the protein treated with active tyrosinase is less than the color of the protein treated with inactive tyrosinase, the reaction is called negative; if the same, it is called positive.

† Digest contained only 2.5 mg. of tyrosine and 0.05 ml. of tyrosinase.

‡ Obtained in crystalline form from the Plaut Research Laboratory. Each enzyme preparation contains about 40 per cent $MgSO_4$.

§ Prepared according to Koch (8).

‖ A saturated solution was used. It was kindly furnished by Dr. I. Fankuchen, the Polytechnic Institute of Brooklyn.

¶ The products of plasma fractionation employed in this work were developed by the Department of Physical Chemistry, Harvard Medical School, Boston, Massachusetts, under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Harvard University. Both the albumin and the γ-globulin are about 98 per cent pure.

** Kindly furnished by the Armour Laboratories. These proteins were prepared under a contract recommended by the Committee on Medical Research, between the Office of Scientific Research and Development and Armour and Company. The albumin is 100 per cent pure; the β-globulin is 98 per cent pure (contains 2 per cent albumin); the fibrinogen is 77 per cent pure (contains 18 per cent globulin plus 5 per cent albumin) and is mixed with 40 per cent sodium citrate.

§§ Kindly furnished by Dr. R. Dubos, The Rockefeller Institute for Medical Research.

in Table I. As would be expected, the substrates which contain no tyrosine, such as gelatin, protamine, and gramicidin, show no evidence of oxidation by tyrosinase.

It is also apparent from Table I that, although many proteins are oxidized, there remain a large number which contain appreciable amounts of tyrosine, but nevertheless are not oxidized under the influence of tyrosinase. In these proteins it is possible that the tyrosine residues are not accessible at the surface of the protein molecule, or the phenolic groups may be so combined as to be unavailable for attack by tyrosinase. With these possibilities in mind it seemed likely that tyrosinase action on these resistant proteins could be demonstrated if the protein molecule were partially
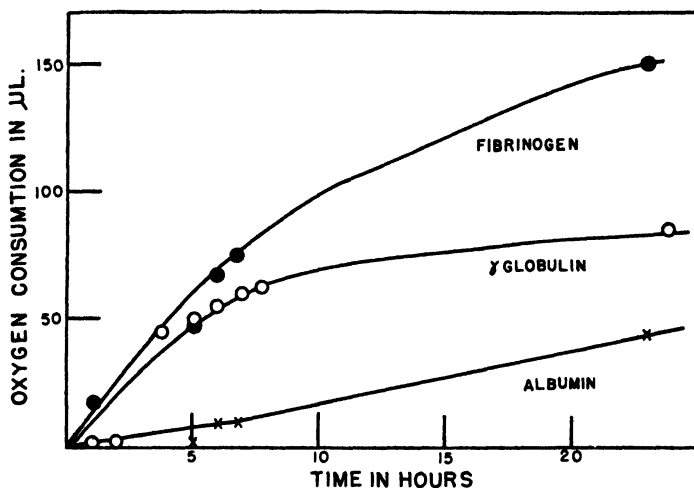
Fig. 2. Oxygen consumption catalyzed by tyrosinase of trypsin-treated proteins. The solution contained 25 mg. of protein, 4.5 ml. of buffer, 1 mg. of cryastlline trypsin, 0.5 ml. of Upjohn tyrosinase, plus 2 drops of toluene.

digested with trypsin before or concurrently with the addition of tyrosinase. That this is possible is shown for several different proteins in Fig. 2, from which it appears that the addition of 1 mg. of crystalline trypsin to the digest rendered the protein oxidizable by tyrosinase. Results of this study are summarized in Table I, which also shows that a substrate such as gramicidin which contains no tyrosine is not rendered oxidizable by tyrosinase after preliminary digestion with trypsin.

Effect of Tyrosinase on Biological Activity of Typical Proteins—Since tyrosinase most readily oxidizes pepsin, trypsin, and chymotrypsin (see Fig. 1), a study was made of the effect of tyrosinase on the enzymatic activity of each of these crystalline enzymes. The technique employed in

studying the proteolytic activities of these enzymes was the same as that described previously (9). It involved the suspension in the proteolytic enzyme solution at 37° of a filament of reprecipitated purified collagen to which was attached a 2 gm. paraffin-coated lead weight. The time required for the filament to break and the weight to fall was measured with an automatic timing device (10). In the first series of tests the enzyme solutions were studied for proteolytic activity at the completion of the manometer experiments in which oxidation of the protein catalyzed by tyrosinase was measured. No change in pH of these solutions was necessary in order to study the activity of trypsin and chymotrypsin, but the measurement of peptic activity required the change in pH from 7.3 (necessary for tyrosinase action) to pH 2.0 by the addition of a few drops of 1 N HCl to the pepsin solution containing boiled or native tyrosinase. In the second series of experiments the procedure was the same except that, in-

TABLE II

Action of Tyrosinase on Activity of Certain Enzymes As Measured by Time Required to Digest Collagen Filament

Protease	Tyrosinase	Digestion time
		hrs.
Chymotrypsin	Boiled	2.16
"	Native	2.10
Trypsin	Boiled	1.32
"	Native	1.54
Pepsin	Boiled	38 Approximately
"	Native	38 "

stead of the manometric technique, the solutions were incubated with native or boiled tyrosinase for 18 hours at 37° before the proteolytic activity was measured. As would be expected, results were very similar by both methods. Typical results are presented in Table II, from which it is apparent that tyrosinase has no effect upon the activity of pepsin, trypsin, or chymotrypsin despite the fact that it catalyzes the oxidation of tyrosine in all three enzymes. These results are surprising in view of the fact that chemical reagents which combine with or oxidize the tyrosine residues of pepsin (11) and chymotrypsin (9) produce inactivation of these proteases. It seems likely that the enzymatic oxidation of tyrosine in proteins does not bring about as drastic a change in the tyrosine moiety as does the chemical oxidation.

Chemical Studies of Effect of Tyrosinase on Proteins—Upon completion of each manometric experiment the digests containing either native or boiled tyrosinase were saved for chemical and spectroscopic examination to de-

termine whether or not the oxidation of the protein molecule induced by tyrosinase could be demonstrated by techniques other than the manometric one.

Studies on Native Protein

Phenol Test for Tyrosine Plus Tryptophane—The phenol reagent was prepared according to Folin and Ciocalteau (12), and a standardization curve was made for tyrosine measuring the absorption at 420 mμ with a Coleman spectrophotometer. The test on the tyrosinase-treated protein solution was made by adding 10 ml. of 0.5 N NaOH plus 3 ml. of phenol reagent to 5 ml. of protein solution. After the solution had stood for 5 minutes, the absorption at 420 mμ was measured. In each experiment the absorption of the protein solution with native tyrosinase was compared with the absorption of the solution containing boiled tyrosinase. No significant difference in the amount of tyrosine plus tryptophane could be detected by this technique, which is not applicable to the study of tyrosine of intact protein molecules before hydrolysis.

Qualitative Millon's Test for Tyrosine—This test was performed by adding to 1 ml. of tyrosinase-treated protein 3 ml. of water plus 3 drops of Millon's reagent (13). The solution was then brought to a boil and allowed to cool. Results of these studies were what might have been predicted (see Table I). In all cases in which oxidation of the protein by tyrosinase was indicated manometrically by oxygen consumption, a much fainter pink color was obtained than for the control solution containing inactive tyrosinase. On the other hand, for those substrates not oxidized under the influence of tyrosinase there was no detectable qualitative difference in color with Millon's reagent between proteins treated with active or inactive tyrosinase. Of course gelatin, protamine, and gramicidin, which contain no tyrosine, gave no color at all with Millon's reagent, regardless of the presence or absence of active tyrosinase. Those proteins resistant to tyrosinase, but rendered labile by digestion with 1 mg. of trypsin, did not react as intensely with Millon's reagent as did the controls treated with inactive tyrosinase. From the results of these studies with Millon's reagent it appears that tyrosinase so modifies the phenol groups of certain labile proteins that they no longer produce an intense pink color in this test.

Quantitative Determination of Tyrosine in Protein Hydrolysates

Bernhart's modification of the Millon-Weiss method for tyrosine determination (14) was used, according to the directions given by Block and Bolling (15). In place of the 10 mg. of protein used in the test, 2 ml. of tyrosinase-treated protein containing 5 mg. of protein per ml. were used. The final reddish colored solution which was obtained showed a maximum

absorption at 420 mμ and so the calibration curve for tyrosine was made at this wave-length. All subsequent measurements on the protein hydrolysates were also made at 420 mμ. Results of the tyrosine analysis by this test are presented in Table III, from which it appears that for tyrosinaselabile proteins the action of tyrosinase results in all cases in a decrease

TABLE III

Effect of Tyrosinase on Tyrosine Content of Proteins As Measured by Modified Millon-Weiss Test for Tyrosine in Protein Hydrolysates

Protein	Tyrosinase	Tyrosine in 10 mg. protein
		mg.
Serum albumin, bovine	Inactive	0.55
" " "	Active	0.59
" " human	Inactive	0.53
" " "	Active	0.61
" " " + trypsin	Inactive	0.80
" " " + "	Active	0.71
Casein	Inactive	0.71
"	Active	0.63
Chymotrypsin	Inactive	0.35
"	Active	0.23
Fibrinogen + trypsin	Inactive	0.57
" + "	Active	0.52
β-Globulin, bovine, + trypsin	Inactive	0.76
" " + "	Active	0.70
γ-Globulin, human, + "	Inactive	0.72
" " + "	Active	0.51
Gramicidin + trypsin	Inactive	0.08
" + "	Active	0.08
Hemoglobin	Inactive	0.36
"	Active	0.18
Insulin	Inactive	1.04
"	Active	0.77
Pepsin	Inactive	0.53
"	Active	0.33
Trypsin	Inactive	0.49
"	Active	0.34
Tyrosine (0.1 mg.)	Inactive	0.08
" (0.1 ")	Active	0.02
Tyrosinase		0.02

(as compared with the control which was treated with inactive tyrosinase) in the amount of tyrosine present in an alkaline hydrolysate of the protein. The results of the chemical tests confirm the manometric studies in reflecting the oxidation of phenolic groups in native proteins catalyzed by tyrosinase.

Effect of Tyrosinase on Color of Proteins

In view of the fact that tyrosinase, in acting on tyrosine, mono- and polyphenols and their derivatives, produces pigmented oxidation products, it is not surprising that proteins oxidized catalytically by tyrosinase are converted to colored end-products. The protein solutions studied mano-metrically for oxygen consumption gradually darkened to a reddish brown color in the presence of active tyrosinase, but not in the presence of boiled tyrosinase. This was true only of those proteins which were oxidized by tyrosinase; no change in color was observed, if the protein was not oxidized. The results of a typical experiment are shown in Fig. 3 for chymotrypsin.
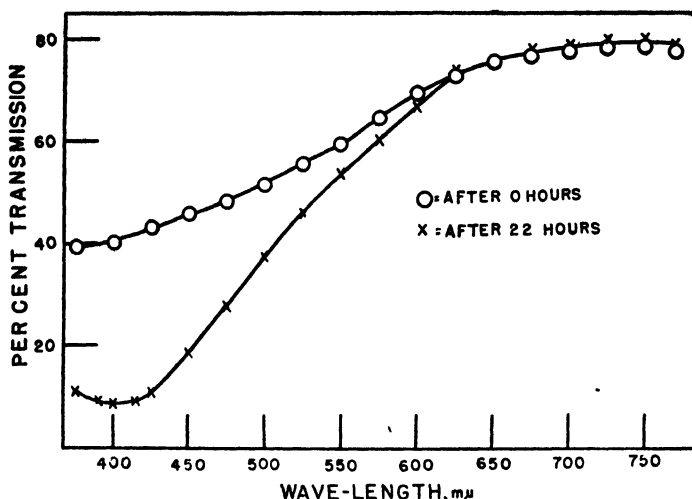

FIG. 3. Effect of tyrosinase on the visible absorption spectrum of crystalline chymotrypsin. The solution contained 25 mg. of chymotrypsin, 4.5 ml. of phosphate buffer, pH 7.3, 2 drops of toluene, and 0.5 ml. of tyrosinase added at zero time. The chymotrypsin is oxidized to a brownish color which absorbs strongly in the blue-violet region.

The upper curve is the transmission curve immediately after adding 0.5 ml. of Upjohn tyrosinase to 25 mg. of crystalline chymytrypsin (in 4.5 ml. of phosphate buffer at pH 7.3). The transmission after 22 hours at 37° is shown by the lower curve. Although with many protein substrates there is some increase at other wave-lengths in absorption produced by tyrosinase, the maximum effect with all proteins is in the violet region at 410 mμ. Similarly the action of tyrosinase on tyrosine results in a maximum absorption in the violet region.

In view of the fact that tyrosinase produces a color change of certain labile proteins, it is possible to use this production of pigment as a means of

studying the kinetics of oxidation of proteins under the influence of ty-
rosinase (see Fig. 4).   A comparison of Figs. 1 and 4 indicates that the ki-
netics of protein oxidation are similar when followed manometrically or
colorimetrically.

Effect of Tyrosinase on Ultraviolet Absorption Spectrum of Proteins

Since the absorption peak characteristic of most proteins at about 276 mμ
can be chiefly ascribed to the presence of tyrosine and tryptophane (16, 17),
it seemed likely that any effect of tyrosinase on the tyrosine of proteins
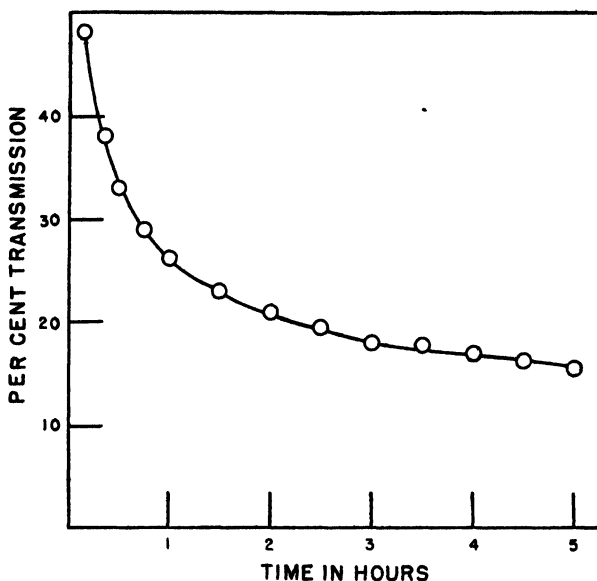
FIG. 4.   Kinetics of the increase in color at 410 mμ associated with the oxidation of
chymotrypsin catalyzed by tyrosinase.   The composition of the solution is the same
as for Fig. 3.

would be reflected in a change in the ultraviolet absorption spectrum,
especially in the region of 276 mμ.

The absorption spectra were taken in the Spectroscopy Laboratory of the
Massachusetts Institute of Technology with a Hilger quartz spectrograph
equipped with a Spekker photometer.   Detailed studies were made with
untreated pepsin, chymotrypsin, and trypsin, and these same proteins
after treatment with tyrosinase for several days.   These solutions had been
studied manometrically before their ultraviolet absorption was measured.
As usual, the protein concentration was 5 mg. per ml, in phosphate buffer
at pH 7.3.   The control solutions contained 0.1 ml. of water per ml., while
the experimental solution contained 0.1 ml. of Upjohn tyrosinase.   The

absorption of the tyrosinase solution was studied independently with 0.1 ml. in 0.9 ml. of buffer. In order to measure the absorption it was necessary to adjust the concentration of protein in solution by dilution with water to different extents, depending on the absorption of the specific protein used. Before plotting the results the log extinction coefficients were multiplied by the dilution factors to put the data for all solutions on a comparable basis.[2]

Results with pepsin are shown in Fig. 5. The control curve for pepsin in buffer agrees closely with that reported by Gates (18, 19) with a maximum absorption at 275 mμ attributable to tyrosine and tryptophane
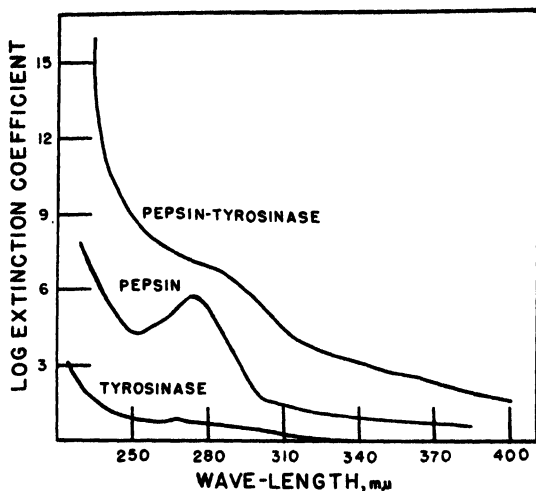

FIG. 5. The ultraviolet absorption spectrum of certain protein solutions. Upper curve, the solution contains 25 mg. of crystalline pepsin, 4.5 ml. of buffer, 0.5 ml. of tyrosinase, 0.001 mg. of merthiolate. Middle curve, the same, but 0.5 ml. of water instead of tyrosinase. Lower curve, 4.5 ml. of buffer, 0.5 ml. of tyrosinase, 0.001 mg. of merthiolate. The oxidation of pepsin by tyrosinase has increased the absorption in the ultraviolet with the elimination of the maximum in absorption at 275 mμ attributable to tyrosine.

The tyrosinase shows a typical protein absorption spectrum with a slight peak (more apparent when the data are plotted on a larger scale) at 268 mμ, agreeing fairly well with the absorption band reported by Dalton and Nelson (20). The oxidation of pepsin catalyzed by tyrosinase increases the absorption throughout the ultraviolet, but the specific peak at 275 mμ attributable to tyrosine has been completely eliminated, suggesting an

[2] This correction involves the assumption that Beer's law applies to the absorption of these solutions in the ultraviolet. Even if it did not strictly apply, the error would not be great, since the dilution factors did not differ appreciably for the different solutions.

oxidation of the tyrosine of pepsin by tyrosinase. Results with chymotrypsin are very similar to those for pepsin. The control solution is characterized by a sharp peak at 272 mμ, a minimum at 253 mμ, and "end-absorption" below 240 mμ. The oxidation of chymotrypsin by tyrosinase gives rise to an increase in absorption over most of the ultraviolet, but the sharp peak in absorption at 272 mμ is eliminated. Tyrosinase also produced an increase in absorption of trypsin over most of the ultraviolet spectrum, but the solutions were not satisfactory for comparison at 280 mμ, at which point trypsin shows a sharp peak in absorption (21, 22).

These studies on light absorption clearly demonstrate that oxidation of typical proteins catalyzed by tyrosinase results in an increased absorption by the protein in the blue-violet and ultraviolet, and that the specific absorption of the protein attributable to tyrosine is greatly decreased.

DISCUSSION

The oxidation by tyrosinase of tyrosine in certain labile proteins or in proteins rendered labile by treatment with trypsin is clearly indicated by studies of oxygen consumption, production of pigment, decrease in color produced with Millon's reagent, decrease in tyrosine in the protein hydrolysate as measured quantitatively by the modified Millon-Weiss method, and increased absorption in the ultraviolet accompanied by the elimination of the peak at 276 mμ attributable to tyrosine. The rate of oxidation of proteins by tyrosinase is very much less than the corresponding rate of oxidation of tyrosine. Although in each case the oxidation is believed to proceed through the formation of dopa (3,4-dihydroxyphenylalanine), the further oxidation of tyrosine is not as extensive for the tyrosyl moiety of proteins as it is for free tyrosine which is converted to melanin (1).

The striking difference in susceptibility of different proteins to attack by tyrosinase suggests that in some proteins the tyrosyl group is available at the surface of the molecule, while in others the stereochemical configuration of the protein is such as to render the tyrosine residue inaccessible to oxidation by tyrosinase. Even in those proteins labile to tyrosinase only a fraction (about 10 to 20 per cent) of the total tyrosine was oxidized by tyrosinase; a large part of the tyrosine appears inaccessible to tyrosinase. On the other hand a very large fraction of the tyrosyl residues of proteins reacts with certain chemical reagents (9, 11, 23, 24). The difference in extent of reaction with tyrosyl groups of proteins by tyrosinase and chemical reagents is also illustrated by the fact that the former does not destroy the biological activity of the proteins, while the latter inactivate the proteins.

In view of the fact that the oxidative enzyme tyrosinase has been shown to act on certain proteins *in vitro*, it seems possible that other non-proteo-

lytic enzymes will also attack native proteins. In particular the action on proteins of such enzymes as deaminases, decarboxylases, dehydrogenases, and certain oxidases deserves careful study. From such investigations may come at least a partial interpretation of the great lability of proteins in living systems.

SUMMARY

Both crude and highly purified mushroom tyrosinases have been demonstrated to oxidize the tyrosyl groups of such proteins as trypsin, pepsin, chymotrypsin, casein, peptone, insulin, and hemoglobin. Tyrosinase did not oxidize gelatin, protamine, and gramicidin, which are devoid of tyrosine. Certain proteins containing tyrosine were resistant to tyrosinase; these included egg albumin, human and bovine serum albumin, tobacco mosaic virus, human γ-globulin, bovine β-globulin, and bovine fibrinogen. All members of this group which were studied were oxidized by tyrosine after a preliminary treatment with crystalline trypsin.

Several techniques were employed in studying the action of tyrosinase on proteins, all of which methods yielded consistent data. These procedures included (1) the measurement of oxidation manometrically from oxygen consumption; (2) the measurement of phenolic groups with Millon's test on intact proteins; (3) the quantitative determination of tyrosine in protein hydrolysates; (4) the quantitative measurement of pigment production in the blue-violet caused by the action of tyrosinase on proteins; (5) the measurement of change in the ultraviolet absorption spectrum especially at $275 \, m\mu$, at which wave-length the protein absorption is due to tyrosine and tryptophane.

In contrast to most chemical reagents which react with the tyrosyl group to destroy the biological activity of the protein, tyrosinase has no effect on the enzymatic activity of pepsin, trypsin, and chymotrypsin. This difference may be related to the fact that only a small fraction of the total tyrosine of the protein is oxidized by tyrosinase, and that each tyrosyl group does not appear to undergo a very extensive oxidation.

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MICROBIOLOGICAL METHODS FOR THE
DETERMINATION OF AMINO ACIDS

III. EXTENSION OF THE UNIFORM ASSAY METHOD FOR THE TEN ESSENTIAL AMINO ACIDS TO INCLUDE TYROSINE

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In an attempt to extend, standardize, and thus simplify microbiological methods for the determination of amino acids, a uniform assay method for the ten essential amino acids in proteins and foods was developed previously (1). One standard medium and one procedure are employed in the method. Nine of the essential amino acids are assayed with *Streptococcus faecalis* and phenylalanine with *Lactobacillus delbrückii* LD5, and the response of the two organisms to the amino acids is measured by titrating, with standard NaOH, the lactic acid produced during growth. A complete analysis can be made with 1.5 gm. or less of sample. An extension of this basic method to include the determination of tyrosine in proteins and foods is described in the present paper.

Procedure

Assays are carried out with *Lactobacillus delbrückii* LD5. Directions for obtaining and maintaining this organism and the preparation of inoculum, assay medium, standard, and samples for assay are exactly as outlined previously (1, 2). Routinely, 0.5 gm. or 1.0 gm. of dried sample is hydrolyzed with 10 cc. of 5 N NaOH in sealed ampules by autoclaving at 15 pounds steam pressure (121°) for 10 hours. This is the procedure previously recommended for the assay of tryptophane (1), and hydrolysates prepared in this fashion can be used for the assay of both amino acids. The precipitate which forms on neutralization of the alkaline hydrolysate is best removed by centrifugation and must be washed twice with about 20 cc. quantities of water to insure complete separation of adhering tyrosine. Assays are titrated with 0.1 N NaOH after 3 days incubation at 37°.

Only the *l* or naturally occurring isomer of tyrosine can be metabolized by *Lactobacillus delbrückii;* the *d* isomer is completely inactive. Identical standard curves are obtained with the *l* and *dl* forms when twice as much of the optically inactive form is used. Since *l*(−)-tyrosine is the more readily available form, it was used exclusively as the standard. The microbial response to increments of tyrosine in the range of 0 to 100 γ is shown in Fig. 1.

Hydrolysis of the sample with alkali under the conditions described above results in complete racemization of the tyrosine.   Since the d isomer does not promote growth, the assay value must be multiplied by a factor of 2, as is the case with tryptophane, to obtain the final tyrosine content of the sample.

RESULTS AND DISCUSSION

Acid or alkali can be used to hydrolyze purified proteins for the determination of tyrosine (3, 4).   However, only alkali may be used to digest proteins contained in natural products, since the accompanying carbohy-
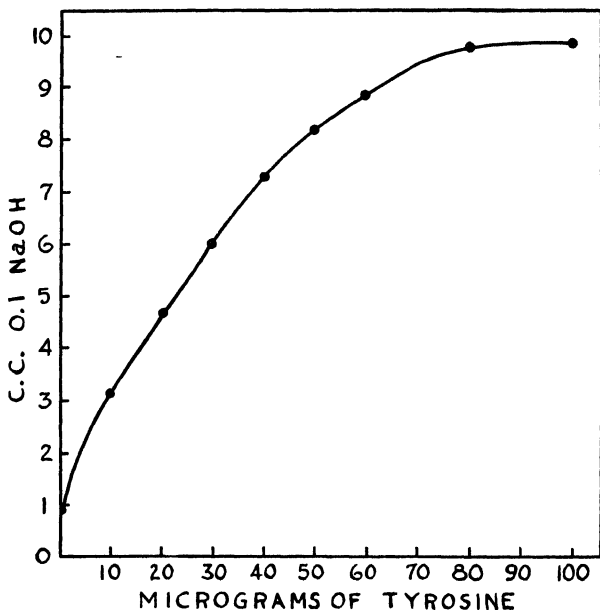
FIG. 1. Standard tyrosine curve

drate may produce considerable loss of tyrosine on acid hydrolysis because of humin formation (5–7).   This is clearly indicated in Table I.   It is noteworthy that loss of tyrosine on acid hydrolysis of foods is appreciable only when the ratio of carbohydrate to protein exceeds 1:1; at a ratio of 7:1 or 9:1 almost 50 per cent of the tyrosine disappears.   Loss of tyrosine due to humin formation is readily demonstrated also by means of synthetic carbohydrate-protein mixtures.   Xylose or arabinose was added, in varying proportions, to casein and blood meal prior to digestion with acid.   As the ratio of carbohydrate to protein is increased from 0.5:1 to 8:1, a progressive loss of tyrosine occurs so that at the highest ratio less than 50 per

cent of the tyrosine is recovered (Table II). Xylose and arabinose behave similarly in this respect and appear to be more active than the carbohydrates in the natural materials. With the latter, destruction of tyrosine is evident only when a 1:1 ratio of carbohydrate to protein is exceeded, whereas with the prepared carbohydrate-protein mixtures at the 1:1 ratio more than 20 per cent loss of tyrosine occurs. Interestingly, free tyrosine is considerably less susceptible to humin formation than tyrosine combined in proteins; only about one-half as much of the former as com-

TABLE I

Tyrosine Content of Proteins Hydrolyzed with Alkali and Acid

The results are calculated in mg per gm of partially dried material.

Protein	5 N NaOH*	10 per cent HCl†	Per cent change with acid hydrolysis	Approximate ratio of carbohydrate to protein (literature reference No.)
Wheat, seed	4 4	2.8	−36	6:1 (8)
Flour, patent	4 3	2.5	−42	7:1 (8)
Barley, seed	5 8	3.6	−38	9:1 (8)
Corn-meal, white	4 8	2 7	−44	9:1 (9)
String bean‡	5 6	2 6	−54	3:1 (8)
Banana‡	1 2	0 53	−56	19:1 (8)
Milk, whole	12 7	10 3	−19	1 4:1 (9)
Yeast, brewers'	15 3	15.6	0	0 8:1 (8)
Egg, whole‡	20	19	−5	0.06:1 (8)
Blood meal	18 4	20	+9	0 03:1 (9)
Beef brain‡	17 3	16 0	−8	0.1:1 (8)
" muscle‡	25	25	0	Low in carbohydrate
Casein	49	51	+4	" " "
Gelatin	3 6	3 3	−8	" " "
Glycinin	31	34	+10	" " "
Egg albumin	32	32	0	" " "
β-Lactoglobulin	32	34	+6	" " "
Tobacco mosaic virus	30	32	+7	" " "

* Autoclaved for 10 hours at 15 pounds steam pressure (121°).
† Autoclaved for 5 hours at 15 pounds steam pressure (121°).
‡ Completely dried material.

pared to protein tyrosine disappeared on acid hydrolysis with added carbohydrate.

In contrast, there is no significant loss of tyrosine on digestion of carbohydrate-protein mixtures in a ratio of 1:1 with NaOH (Table III). Also, no loss of tyrosine occurred when the ratio of carbohydrate to protein was increased to 3:1. Similar results were obtained by Lugg with free tyrosine and edestin (7). Digestion with alkali was adopted, therefore, for routine use in preparing natural materials for assay.

Autoclaving of proteins at 15 pounds steam pressure (121°) for 5 hours with 5 N NaOH is adequate for complete liberation and racemization of tyrosine (Table IV).   There are indications with casein and wheat that prolongation of the heating time to 30 hours results in some destruction of

TABLE II

Effect of Added Carbohydrate on Tyrosine Content of Proteins Hydrolyzed with Acid

The results are calculated in mg. per gm. of partially dried material.

Protein	Carbohydrate added		Ratio of carbohydrate to protein					
			0.25:1	0.5:1	1:1	2:1	4:1	8:1
Casein	None	52						
	l-Xylose		48 (7.7)*	46 (12)	40 (23)	36.5 (30)	33 (37)	25.5 (51)
	l-Arabi-nose		48 (7.7)	45 (14)	41 (21)			
Blood meal	None	20						
	l-Xylose		17.6 (12)	16.6 (17)	15.4 (23)	13.8 (31)	11.9 (40)	9.6 (52)
	l-Arabi-nose		17.4 (13)	17.0 (15)	14.6 (27)			
l-Tyro-sine	None	24.8						
	l-Xylose			22.3 (10)	21.8 (12)	21.3 (14)	20 (19)	18.1 (27)
	l-Arabi-nose			22.5(9.3)	22 (11)			

* The figures in parentheses are per cent decreases of tyrosine.

TABLE III

Effect of Added Carbohydrate on Tyrosine Content of Proteins Hydrolyzed with Alkali

The results are calculated in mg. per gm. of partially dried material.

Protein	Carbohydrate added*		
	None	l-Xylose	l-Arabinose
Casein........................	48	49 (+2.1)†	50 (+4.2)
Blood meal...............	18.2	18.4 (+1.1)	19.0 (+4.4)
Glycinin, soy bean........ ...	32	30 (−6.3)	31 (−3.1)
Yeast, brewers'......	16.3	16.0 (−1.8)	16.8 (+3.1)
Beef brain‡	19.8	17.4 (−12)	21.8 (+10)

* An amount equal to that of protein was used.

† The figures in parentheses are per cent changes in tyrosine.

‡ Completely dried material.

tyrosine.   Otherwise this amino acid appears to be quite resistant to heating with alkali.   Hydrolysis of casein, blood meal, and yeast with 10 per cent HCl is also complete in 5 hours.

Although autoclaving for 10 hours with 5 N NaOH is used routinely,

2 N NaOH appears to be sufficient for complete digestion (Table V). Also, ·Ba(OH)₂, under the conditions described by Greene and Black (10), can be used in place of NaOH.

'The reliability of the microbiological tyrosine values is supported by their good reproducibility on repeated assay (Table VI) and by the quan-

TABLE IV

Effect of Time of Hydrolysis with Alkali and Acid on Liberation of Tyrosine from Proteins

The results are calculated in per cent of partially dried material.

	Hydro-lyzing agent	Hrs. of hydrolysis*					
		2	5	10	15	20	30
Casein...................	NaOH†		5.4	5.2	5.1	4.9	4.6
	HCl‡	5.0	5.0	4.8		4.8	
Blood meal..............	NaOH		1.93	1.85	1.91	1.85	1.87
	HCl	1.83	2.00	1.90		2.00	
Yeast, brewers'..........	"	1.65	1.58	1.59		1.56	
Glycinin, soy bean.......	NaOH		3.2	3.0	3.0	2.9	2.9
Beef brain§.............	"		1.53		1.59	1.51	1.59
Wheat, seed.............	"		0.44	0.40	0.40	0.39	0.35

* Autoclaved at 15 pounds steam pressure (121°).
† 5 N NaOH.
‡ 10 per cent HCl.
§ Completely dried material.

TABLE V

Effect of Concentration and Type of Alkali on Liberation of Tyrosine from Proteins

The results are calculated in per cent of partially dried material.

Protein	NaOH*				Ba(OH)₂ *
	1 N	2 N	3 N	5 N	6 N
Corn-meal.................	0.40	0.48	0.44	0.49	0.51*
Wheat, seed................	0.39	0.44	0.45	0.47	0.42*
Casein....................				5.2	5.0†

* Autoclaved for 10 hours at 15 pounds steam pressure (121°).
† Autoclaved for 7 hours at 15 pounds steam pressure (121°).

titative recovery, within 10 per cent, of tyrosine added to proteins prior to hydrolysis (Table VII). In the recovery experiment it was necessary to autoclave the mixtures containing 0.5 gm. of protein and 5 mg. of tyrosine for 30 hours to obtain complete racemization of the added tyrosine. The well known fact that amino acids with a free carboxyl group are difficult to

racemize (11) is demonstrated clearly in the case of tyrosine. Although 5 mg. of tyrosine combined in proteins can be completely racemized in 5

TABLE VI

Reproducibility of Tyrosine Values

The results are calculated in per cent of partially dried material.

Protein*	Assay 1	Assay 2	Assay 3	Assay 4	Mean
Casein......................	4.8	4.8	5.2	4.9	4.9
Glycinin, soy bean..........	3.2	3.0	3.3	2.9	3.1
Blood meal.................	1.82	1.85	2.1	1.85	1.91
Corn-meal..................	0.49	0.47	0.49		0.48
Flour, patent.......	0.47	0.42	0.40		0.43

* Autoclaved with 5 N NaOH for 10 or 20 hours at 15 pounds steam pressure (121°)·

TABLE VII

Recovery of Tyrosine Added to Proteins Prior to Hydrolysis* with 5 N NaOH

The results are calculated in mg. per gm. of partially dried material. 10 mg. of tyrosine were added to each substance.

Substance	Content	Total	Found	Per cent recovery
Casein	48.8	58.8	58.4	99
Linseed meal	9.4	19.4	19.7	101
Beef brain†..	19.2	29.2	26.2	90
Blood meal.	18.4	28.4	31.4	110

* Autoclaved for 30 hours at 15 pounds steam pressure (121°).
† Completely dried material.

TABLE VIII

Influence of Time on Racemization of Tyrosine with 5 N NaOH

l(−)-Tyrosine	Hrs. of hydrolysis*			
	5	10	20	30
	Per cent tyrosine recovered			
mg.				
5	170	144	114	100
10		152	124	115

* Autoclaved at 15 pounds steam pressure (121°).

hours with 5 N NaOH (Table IV), it is necessary to heat 5 mg. of free tyrosine for 30 hours to obtain the same effect (Table VIII).

The response of *Lactobacillus delbrückii* to tyrosine is quite specific. The

following compounds were less than 1 per cent as active as tyrosine on a weight basis: tyramine, epinephrine, phenylacetic acid, mono-N-phenyl-acetylethylenediamine hydrochloride, N-phenylacetylethanolamine, and α-bromo-β-phenylpropionic acid.   Phenylalanine, which is a constituent of the basal medium, is, of course, also inactive.   It is of interest and also

TABLE IX

Comparison of Microbiological Values of Tyrosine with Those Cited in Literature

Substance	Per cent nitrogen (dry basis)	Per cent tyrosine (dry basis)	Per cent tyrosine in protein (N × 6 25) on dry basis		
			Micro-biological values	Literature values	Bibliographic reference No
Wheat, seed	2 22	0 49	3 5	3 9, 4 8	(13)
Flour, patent	2 28	0 49	3 4	3 8	(13)
Rye, seed	1 95	0 41	3 4	4 8	(13)
Barley, seed	2 59	0 63	3 9		
Corn-meal, white	1 65	0 53	5 1	5 6,* 7 1*	(13)
•Soy bean flour, defatted	9 32	2 1	3 6	4 1	(13)
Alfalfa meal	2 90	0 64	3 5	5 7	(13)
Yeast, brewers'	9 14	1 64	2 9	3 6, 4 2	(13)
Milk, whole	4 34	1 37	5 0	5 0, 5 5	(13)
Egg, "	7 97	2 0	4 1	4 2, 4 3	(13)
String bean	2 86	0 56	3 1		
Banana	0 75	0 12	2 6		
Beef muscle	11 90	2 5	3 4	2 2, 3 4, 4 3	(13)
" brain	7 38	1 73	3 8	4 6, 4 8	(13)
Blood meal	14 96	2 0	2 2	2 0†	(13)
			Per cent of dry weight		
Casein, S M A ‡		5 6		5 3, 6 01, 6.37	(13–15)
Gelatin, Knox‡		0 41		0 3, 0 26, 0 46	(13, 16, 17)
Glycinin, soy bean‡		3 7		4 55	(18)
Egg albumin‡		3 4		3 93, 3 86, 3 89	(4, 19, 20)
β-Lactoglobulin‡		3 6		4 2, 3.78	(21, 22)
Tobacco mosaic virus‡		3 4		3 8, 3 9	(23, 24)
Bovine serum albumin‡		5 3		5 53	(25)

* For whole corn
† For cattle hemoglobin
‡ Autoclaved with 10 per cent HCl for 5 hours

essential for the assay method that *Lactobacillus delbruckii* is less versatile than the rat, which can synthesize tyrosine from the phenylalanine supplied to it (12).

It is apparent from Table IX that, in general, the tyrosine values obtained by microbiological assay for the various natural substances and purified

proteins approximate values obtained with chemical methods on similar substances cited in the literature. However, the microbiological figures are almost consistently 10 to 15 per cent lower than those obtained by colorimetric methods. This difference is especially evident with purified proteins. Part or all of it may be due to differences in the samples themselves. Thus the glycinin (Illini) preparation of Vickery, which contains 3.7 per cent tyrosine by microbiological assay, has a nitrogen content of 16.93 per cent compared to 17.74 per cent nitrogen for the glycinin (Illini) of Csonka and Jones, for which they obtained a value of 4.55 per cent tyrosine by the colorimetric method of Folin and Ciocalteu. Also, for some proteins, the chemical values have been corrected for ash and, in the case of lactoglobulin, for estimated losses of tyrosine in the assay. Such corrections have not been made for the microbiological values, since it is not certain that significant losses of tyrosine occurred during hydrolysis of the proteins; corrections for ash content would only slightly alter the microbiological values. The extensive controversy concerning the accuracy of the various colorimetric procedures for measuring tyrosine (4, 26, 27) indicates that values obtained by such methods cannot be considered as unqualified standards in judging the accuracy of the microbiological data. It may be significant that the microbiological value of 5.3 per cent tyrosine (average of 5.1, 5.5, 5.4 per cent) for bovine serum albumin is in good agreement with the figure of 5.5 per cent obtained on the same sample by the accurate isotope dilution methods.

The purified proteins were assayed for tyrosine also with *Streptococcus faecalis* (1) and essentially the same results as with *Lactobacillus delbrückii* were obtained. There was a trend with both organisms towards slightly higher tyrosine values when the proteins were hydrolyzed with 10 per cent HCl for 5 hours compared to hydrolysis with 5 N NaOH for 10 hours. Also, somewhat better agreement at the different assay levels was obtained with the acid hydrolysates, especially in the case of bovine serum albumin. It may be preferable, therefore, to digest purified proteins with acid for microbiological tyrosine assays. Since digestion with acid does not racemize the tyrosine in the protein, assay values must *not* be multiplied by the factor of 2, as is done when alkaline hydrolysis is employed. However, alkaline hydrolysis must be used with natural substances, except possibly for those materials which have a ratio of carbohydrate to protein of less than 1:1, since hydrolysis with acid would destroy tyrosine because of humin formation.

Streptococcus faecalis was used as the assay organism in the initial experiments, but was discarded because growth was frequently inhibited by toxic substances in alkaline hydrolysates of wheat, rye, corn-meal, flour, and other materials which are low in tyrosine and must be added, there-

fore, in relatively large amounts to the assay tubes. Unsuccessful attempts to eliminate the toxicity of these hydrolysates included digestion with NaOH for 15 to 30 hours in place of the customary 10 hours, use of 1, 2, .and 3 N NaOH instead of 5 N alkali, substitution of $Ba(OH)_2$ for NaOH, and increase in incubation from 2 days to 3 or 4 days. The possibility that the toxicity was due to excess NaCl in the hydrolysates which formed on neutralization of the digests was excluded. Substitution of *Lactobacillus delbrückii* for *Streptococcus faecalis* eliminated the difficulty. However, an occasional sample will partially inhibit acid formation also by *Lactobacillus delbrückii*. This occurs usually at the highest assay level of 5 cc. and can be taken care of either by omitting the easily recognized incorrect value from the average or by repeating the assay with smaller quantities of the hydrolysate of the sample. With the exception of barley, corn-meal, egg, string bean, banana, beef muscle, beef brain, glycinin, and bovine serum albumin, which were used for the first time, the substances listed in Table IX are the same preparations assayed for the ten essential amino acids (1).

The authors are greatly indebted to Dr. H. B. Vickery for the glycinin preparation and to Dr. D. Shemin for the sample of bovine serum albumin.

SUMMARY

The previously developed basic microbiological assay method for the determination of the ten essential amino acids in proteins and foods has been extended to include the assay of tyrosine. The method is based upon the quantitative response of *Lactobacillus delbrückii* LD5 to increments of 0 to 100 γ of tyrosine as measured by titration with 0.1 N NaOH of the lactic acid produced during growth. The reliability of the method is supported by the good agreement of values for different amounts of sample assayed, reproducibility of values on repeated assay, and by the quantitative recovery, within the usual microbiological variation of 10 per cent, of tyrosine added to proteins prior to hydrolysis. As much as 50 per cent of the tyrosine in foods with a high carbohydrate content may be lost owing to humin formation if hydrolysis with acid is employed. Such losses do not occur when hydrolysis is carried out with alkali. This procedure is accordingly routinely applied to all natural materials. Either acid or alkali may be used to hydrolyze purified proteins. The microbiological tyrosine values for the fifteen natural materials and seven purified proteins assayed are in fair agreement, in most instances, with chemical values on similar substances cited in the literature.

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THE SYNTHESIS OF ISOCITRIC ACID

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Present day interest in the rôle of isocitric acid in the metabolism of many animal and plant tissues has created a need for this somewhat rare substance. The naturally occurring optically active isomer of isocitric acid is, of course, desirable for use as a substrate for the investigation of enzyme reactions. Work is now in progress with the object of simplifying the preparation of this isomer from the leaves of certain members of the family Crassulaceae, in particular from the leaves of *Bryophyllum calycinum*, in which it is normally present to the extent of 8 to 10 per cent or more of the dry weight (1, 2). Nevertheless the racemic acid is useful for many purposes and attention has accordingly been given to the synthesis of this substance.

Three methods for the synthesis of isocitric acid or of its lactone have been described. The method of Fittig and Miller (3) depends upon the condensation of chloral with sodium succinate in the presence of acetic anhydride and subsequent hydrolysis of the resulting trichloromethylparaconic acid to isocitric acid; this is isolated as the lactone. The method of Wislicenus and Nassauer (4) depends upon the condensation of diethyl oxalate and diethyl succinate to oxalosuccinic ester which is reduced with sodium amalgam to triethyl isocitrate; saponification and dehydration lead to the lactone. The third method is that of Greenstein (5, 6) who obtained isocitric lactone from α-aminotricarballylic acid after treatment with nitrous acid and dehydration; this was not advocated as a practical method for synthesis in quantity.

The method of Fittig and Miller was employed by Nelson (7) and by Krebs and Eggleston (2). Martius (8) also prepared synthetic isocitric acid, but the method he used was not mentioned.[1] Martius and also Krebs and Eggleston observed that 50 per cent of their synthetic isocitric acid reacted when treated with aconitase; it is to be inferred, therefore, that their preparations contained only the naturally occurring acid and its enantiomorph. This is surprising, for inasmuch as isocitric acid contains two centers of asymmetry the synthetic process would be expected to give rise to two racemic diastereoisomers. One of these, *dl*-isocitric acid,

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[1] In addition, Martius prepared optically active isocitric acid, although in poor yield, by the action of liver aconitase upon *cis*-aconitic acid. He likewise states that he succeeded in obtaining small amounts from citric acid by the same means.

should behave as Martius and Krebs have observed; the other, which may be referred to as *dl*-alloisocitric acid, should consist of a pair of enantiomorphs neither of which corresponds to the natural substance.

In this connection, it is significant that the yield reported by Krebs and Eggleston was only 60 per cent, and that Martius remarked that several recrystallizations were required to secure a product of satisfactory melting point. Thus in both cases there is a possibility that alloisocitric acid may have been present in the reaction mixtures but was removed during the purification.

Fittig and Miller Method

In the present study of the synthetic method of Fittig and Miller, it was found that the hydrolysis of trichloromethylparaconic acid with barium hydroxide gives isocitric acid to the extent of 95 per cent, as determined by titration. From 97 to 99 per cent of this could be isolated as substantially pure lactone, as was revealed by titration before and after opening the ring with warm dilute alkali. Nevertheless fractional crystallization of the lactone from ethyl acetate gave only from 80 to 85 per cent of material of a melting point of 162–163°, which was unchanged on recrystallization. From the mother liquors, a much more soluble product was secured in a yield of about 7 per cent of the lactone subjected to fractionation. Although titration of this fraction yielded values in good agreement with that calculated for pure isocitric lactone, the preparation gave every evidence of being a mixture. It melted over the range 135–138° and yielded derivatives that were also mixtures. Recrystallization of the derivatives, however, gave products in small yield that corresponded in properties with those obtained from the main lot of lactone. These were separated from larger proportions of what were clearly derivatives of an isomer of isocitric lactone. The *p*-bromophenacyl esters were particularly satisfactory for this separation. The main component of the fraction thus appeared to be alloisocitric lactone. This substance was not itself obtained in a satisfactorily pure state; several different derivatives were, however, successfully purified, and in all cases mixtures of the respective derivatives of the racemic diastereoisomers melted at depressed temperatures.

In general, the preparation of the derivatives from mixtures presumed to be rich in alloisocitric acid or its lactone was far more difficult than from the purified crystalline lactone secured as the main product of the Fittig and Miller method of synthesis. The derivatives of alloisocitric lactone (or acid) separated slowly and in poor yield and were, as a rule, considerably more soluble in the solvents employed than were the derivatives of the other isomer.

Wislicenus and Nassauer Method

These conclusions respecting the nature of the preparations of isocitric lactone obtained by the Fittig and Miller method were supported by observations made in the course of a study of the Wislicenus and Nassauer method. Triethyl isocitrate was obtained in approximately 89 per cent yield by the reduction of oxalosuccinic ester, and hydrolysis gave a quantitative yield of isocitric acid, as measured by titration. The behavior of the product that resulted after conversion of the ester through the barium salt to the lactone was, however, quite different from that synthesized by the Fittig and Miller method. The viscous sirup failed to deposit crystals even on long standing, although titration before and after treatment with warm alkali showed it to contain at least 95 per cent of the lactone, the balance being isocitric acid. Finally, by treatment with a relatively small proportion of ethyl acetate, a colorless solid which melted at 148–149° was obtained in about 50 per cent yield. Recrystallization from ethyl acetate and toluene converted about two-thirds of this material into a product that melted at 162–163° and corresponded in all properties with the main product obtained by the Fittig and Miller method.

The mother liquors were subjected to an elaborate fractional crystallization process. After the separation of a few negligibly small crops of crystals which were apparently rich in the isomer mentioned above, as evidenced by melting points such as 148° and 150°, somewhat less than half of the lactone present in the solution was obtained as a fraction that melted at 134–137°. The yield of this product was thus about 20 per cent of the total lactone taken. By recrystallization from ethyl acetate-toluene mixtures, a quantity that corresponded to about 8 per cent of the lactone subjected to fractionation was separated as a product that melted at 135–138°. This resembled the small fraction of similar melting point range, rich in alloisocitric lactone, obtained by the Fittig and Miller method; there was no depression of the wide melting point range when the two products were mixed. Furthermore, the *p*-bromophenacyl ester yielded, on recrystallization, a product identical in melting point with that of alloisocitric lactone and mixtures of the two preparations showed no depression in melting point.

These observations permit the conclusion that both the Fittig and Miller and the Wislicenus and Nassauer methods of synthesis of isocitric acid lead, as might be anticipated, to mixtures of both racemic varieties. It fortunately happens, however, that the mixture obtained by the Fittig and Miller method consists very largely, possibly to the extent of 85 per cent, of the isomer which is the racemic form of the acid that occurs in nature. On the other hand, as the mixture obtained by the Wislicenus and Nassauer method contains the two isomers in more nearly equal

proportions, their separation is difficult and the yields of the desired product are necessarily low. No satisfactorily pure specimen of alloisocitric lactone was secured, although small specimens of derivatives of it were isolated in pure form.

A number of observations in the literature on the behavior of synthetic isocitric lactone can be accounted for in the light of these conclusions. The melting points of the products obtained by those who have employed the Fittig and Miller synthesis (160–161° in the original paper, the same in Nelson's, 163° in Krebs and Eggleston's, 164° in Martius', and 162–163° in the present work) are consistent and are to be contrasted with that (120–130°) recorded by Wislicenus and Nassauer and with the value 148–149° for the first crop of lactone obtained by their method in the present work, as well as with those of several fractions separated during the recrystallization of the product. It seems evident that investigators who have employed the Fittig and Miller method have in fact obtained homogeneous material after sufficient purification, and the statements by Martius and by Krebs that the products reacted to the extent of exactly 50 per cent under the influence of aconitase are thus comprehensible. Martius' statement suggests indeed that he made use of the Fittig and Miller method for the synthesis of his preparation.

Although Nelson did not prepare the lactone from the ester he synthesized by the Wislicenus and Nassauer method, he did prepare the trihydrazide. His statement that this "has not been obtained in a state of absolute purity" indicates that difficulties were encountered in the characterization.

The pure lactone of optically active naturally occurring isocitric acid melts at 153–154° (1, 9), about 10° lower than the purified synthetic racemic variety. It may accordingly be inferred that the specimen of synthetic lactone secured by Greenstein (6) from α-aminotricarballylic acid, and which he recorded as melting at 153°, was probably a mixture of the two diastereoisomers which chanced to melt at the same temperature as the lactone of the natural acid. It is to be noted that the yield of lactone he obtained from the barium salt of the synthetic acid was only 45 per cent (0.6 gm. from 3 gm. of anhydrous barium salt). This fact, together with the depressed melting point of the product, suggests that isocitric lactone obtained by Greenstein's method of synthesis is probably a mixture of the isomers in a proportion not unlike that given by the Wislicenus and Nassauer method.

Derivatives of Isocitric Acid

Hydrazides—The trihydrazide of isocitric acid has not proved in the past to be particularly useful for the characterization of isocitric acid

whether of the optically active natural isomer (1) or of the mixture of isomers obtained by synthesis (7). There is still considerable uncertainty regarding the correct melting point of the trihydrazide of the natural acid, values of 201–202° (9), 196–197° (7), and 195–196° (1) having been recorded. These are, however, unusual observations; most preparations obtained in practice from natural sources melt within a few degrees of 181°.

The trihydrazide obtained from the ester synthesized by the Fittig and Miller method melted at 199–201°. It separated promptly and in almost quantitative yield, behaving in general like the trihydrazide of the natural isomer.

The trihydrazide obtained from the ester synthesized by the Wislicenus and Nassauer method separated slowly in a yield that rarely exceeded 30 per cent and melted at 180–185°. By spontaneous evaporation of the mother liquor, a second crop of hydrazide was secured which melted at 194–197°. A mixture of this second crop with the product secured by the Fittig synthesis melted at 185–188°. The evidence points again to the complexity of the mixture of substances obtained by the Wislicenus method, but the instability of the trihydrazides on recrystallization rendered purification difficult. The use of the hydrazides for characterization was accordingly abandoned.

p-Bromophenacyl Esters—Of the several derivatives of isocitric acid that have been examined, the *p*-bromophenacyl esters (10) have proved to be the most useful for the separation and identification of the isomers. The di-*p*-bromophenacyl ester of *dl*-isocitric lactone is readily obtained in a yield of about 64 per cent; it is nearly insoluble in hot absolute alcohol and melts at 189–190°. The corresponding compound of *dl*-alloisocitric lactone was secured in a yield of about 46 per cent from the impure preparations of the lactone obtained by the Fittig and Miller method. This substance is moderately soluble in hot alcohol and accordingly can be separated by extraction from the small quantity of the *dl*-isocitric lactone derivative that was present in the preparation. It separated in pure form when the solvent was cooled, as shown by the fact that the melting point of 153–154° was not changed by further recrystallization. These derivatives thus permit clear and positive differentiation of the isomeric lactones. Mixtures of the two products melt to turbid oils at temperatures intermediate between the melting points of the individual substances.

Substituted Benzyl Pseudothiouronium Salts—S-Benzyl pseudothiouronium chloride (11) did not yield a satisfactory compound with isocitric lactone but the *p*-chloro derivative (12) of the reagent gave in 73 per cent yield a salt which melted at 167–168° after recrystallization. The preparation rich in alloisocitric lactone, however, gave oily products which

could be induced to crystallize with difficulty. After purification, this compound also melted at 167–168°, but a mixture in equal proportions with the corresponding isocitric acid derivative melted at 160–161°. In a further attempt to find a useful reagent for the characterization of the two lactones, p-nitrobenzyl pseudothiouronium chloride was prepared essentially as described by Donleavy (11). A well crystallized salt with a melting point of 153–155° was obtained from isocitric lactone with this reagent. Alloisocitric lactone on the other hand yielded an oil that deposited crystals only after long standing. The crude product decomposed at 176° but could be separated by means of alcohol into unchanged reagent and substances that decomposed between 150–160°. None of the derivatives of this type therefore held promise of serving for the separation of the isomers of isocitric lactone.

p-Toluidine Salts—When isocitric lactone dissolved in ethyl acetate is treated with p-toluidine dissolved in toluene, a crystalline salt promptly precipitates. A monosalt is formed if 1 mole of base is added, a disalt in the presence of 2 or more moles. The monosalt separates in a yield of about 90 per cent and can be recrystallized from alcohol. After being dried *in vacuo*, it melts at 144° and decomposes at 146–148°. The disalt separates in even higher yield but is difficult to obtain uncontaminated by the monosalt. After recrystallization from ethyl acetate in the presence of excess toluidine, the preparation melted with decomposition at 155–156°. When recrystallized without the addition of toluidine, the chief product was the monosalt.

A preparation of the disalt of alloisocitric lactone was obtained in a similar manner but the crystals separated slowly and the yield was not greater than 57 per cent. After being recrystallized, the compound melted at 133° and decomposed at 140°. A mixture of equal parts of the isomeric disalts melted at 127° and decomposed at 131°.

Although these toluidine salts have no especial value for precise characterization or for the separation of the isomers, the disalt has potentialities for the purification of isocitric lactone on the large scale because of the high yield that can be obtained. The lactone can be recovered from the salt by extraction with ether under suitable conditions.

Trichloromethylparaconic Acid

The presence of widely different proportions of isocitric and alloisocitric acids in the material obtained by the Fittig and Miller method raises the question of the homogeneity of the preparation of the intermediate trichloromethylparaconic acid. The tests that have been made by means of the melting points of successive fractions obtained in the course of recrystallization from water gave no evidence of significant separation of

isomers.   Although the final crop, amounting to about 4 per cent of the sample taken, melted at a somewhat depressed temperature, titration indicated the presence of a contaminant with a lower neutralization equivalent, possibly the dicarboxylic acid formed by the opening of the lactone ring during the evaporation of the mother liquors.

The p-bromophenacyl derivative was an oil which failed to crystallize. The p-chlorobenzyl pseudothiouronium salt, however, had excellent properties; the yield from the first crop of trichloromethylparaconic acid was 88 per cent and the melting point, after recrystallization, was 134–134.5°; analysis, N 6.32, theory for $C_{14}H_{14}O_4N_2SCl_4$, N 6.25 per cent. The final crop of trichloromethylparaconic acid, which was presumably somewhat impure, gave the derivative in 75 per cent yield and with the same melting point after being recrystallized; an equal mixture of the two preparations melted at 131–132°.   This small depression could not be regarded as conclusive proof of the presence of isomers.

The instability of trichloromethylparaconic acid suggested that the demonstration of inhomogeneity would be a matter of some difficulty. A study of the solubility has not been attempted.

<div align="center">EXPERIMENTAL</div>

Sodium Succinate—The hexahydrate was prepared by adding a slight excess (334 ml.) of 18 N sodium hydroxide to 354 gm. (3 moles) of succinic acid dissolved in 600 ml. of water.   After filtration and neutralization to phenolphthalein by the addition of sufficient powdered succinic acid to the hot solution, 797 gm. (98.5 per cent) of crystals were obtained in successive crops by evaporation of the mother liquors.   The salt was dehydrated to constant weight at 105–110°, rather than at 140° as recommended by Fittig and Miller.

Trichloromethylparaconic Acid—Study of the details of the synthesis as described by Fittig and Miller[2] resulted in a number of changes that

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[2] Fittig and Miller recommended a reaction temperature of 110–120° and a heating period of 3 to 4 hours.   They considered the reaction to be complete at the time the mass becomes black and suddenly expands in volume.   In the present work, low yields were obtained at temperatures below 120° even when the time was prolonged to 6 hours.   The evolution of gas was not a satisfactory criterion of the end of the reaction, yields of only about 30 per cent being obtained if isolation was attempted at this stage.   Because of the strong tendency to form emulsions, the separation of the greater part of the acid by direct crystallization was a marked improvement over extraction with ether, as practiced by Fittig and Miller.   The recovery of the small residual quantities of acid in the mother liquor by ether extraction then presented no difficulty.   Hydrochloric acid is to be preferred to sulfuric acid for the acidificatioh, as sodium sulfate may become a troublesome contaminant.   Recrystallization from carbon tetrachloride rather than from water is more satisfactory, since both succinic

contributed greatly to the yield and purity of the product. The most advantageous procedure found was as follows: A mixture of 50 gm. of powdered anhydrous sodium succinate and 48 gm. of anhydrous chloral in a 500 ml. flask was rapidly stirred mechanically at room temperature while 31.5 gm. of acetic anhydride were slowly added. A reflux condenser was attached and the oil bath surrounding the flask was then heated to a temperature of 140° during 15 minutes and subsequently maintained within 2° of this temperature, efficient stirring being continued. A colorless solid soon separated and the mass began to darken. After about 30 minutes, a sudden evolution of gas occurred. Heating was continued for a total of 65 minutes from the time the bath reached 140°. The black mixture was cooled to 105° and 200 ml. of hot water were slowly added. The tarry mass was broken up with a rod and heated on the steam bath until it was largely in solution, the extract was decanted, and the residual tar was dissolved by treatment with successive portions of hot water. The final solution, which amounted to 600 ml., was heated with 30 gm. of norit and filtered hot, 700 ml. of hot water being used to wash the norit. The clear pale brown solution was concentrated *in vacuo* to dryness with the aid of a few drops of Turkey-red oil to control frothing. The residue of sodium salt was dissolved as far as possible in 200 ml. of hot water and 60 ml. of concentrated hydrochloric acid were added. The solution was chilled in an ice bath and stirred during the crystallization of the acid. After being chilled overnight, the crystals were filtered, washed with 250 ml. of ice water, and dried in air; yield about 51 gm., melting point 97°. The mother liquor was shaken successively with 150, 100, and 70 ml. quantities of ether, and the combined extracts were washed five times with a little water. The ether was distilled and the residual oil was dissolved in the minimal amount of hot water; 4.6 gm. crystallized, melting point 97°. The total yield was 73 per cent of the theoretical amount; in similar experiments in which unchanged succinic acid was recovered, it corresponded to 81 to 86 per cent of the succinate consumed. On recrystallization from carbon tetrachloride (10 ml. per gm.) with the use of norit a 96 per cent recovery was secured of a product of melting point 95–97°; analysis, Cl 43.0, theory for $C_6H_5Cl_3O_4$, Cl 42.98 per cent. It was necessary to dry the crystals in a current of air heated to 70° to free them completely from the solvent.

Barium Isocitrate—218 gm. of hydrated barium hydroxide (about 10 per cent excess over theory) were dissolved in 400 ml. of hot water and

acid and sodium chloride remain insoluble, and there is no tendency towards the formation of an oily product. The solubility of trichloromethylparaconic acid in carbon tetrachloride is about 8 per cent at boiling temperature and is less than 0.4 per cent in the cold.

filtered into a 1 liter flask in an oil bath heated to 90–95°, and the residue of barium carbonate was washed with 100 ml. of hot water. The stirring mechanism was started and 50 gm. of trichloromethylparaconic acid were added at such a rate that no violent frothing occurred. This operation required about 20 minutes. The bath was then heated to 120° and maintained at this temperature for 2.5 hours with constant stirring. The hot solution was filtered[3] and the barium salt was washed with 700 ml. of boiling water, suspended twice successively in 35 per cent alcohol and then in strong alcohol, and was finally washed with ether. After being dried in air, the product weighed 80.8 gm.

The salt that separates under the conditions described is a monohydrate and it is invariably contaminated with a trace of barium chloride. If a more dilute solution of barium hydroxide is employed for the hydrolysis, the salt has the composition of a tetrahydrate. The monohydrate is obtained in a yield of about 98 per cent after correction for the barium chloride in the preparation; the yield of the higher hydrate is somewhat less owing to its greater solubility. The higher hydrate loses 1 molecule of water at 105°; the monohydrate is stable at this temperature; both salts can be dehydrated at 170°. All preparations examined were somewhat low in barium, the average of five lots being 51.6 per cent instead of the theoretical 52.16 per cent for the anhydrous salt. The barium chloride content of the preparations, calculated as the dihydrate from a chloride titration, ranged from 0.3 to 1.0 per cent.

Isocitric Lactone (Mixture of Isomers)—Barium isocitrate monohydrate equivalent to 315 gm. of anhydrous salt was added slowly to a rapidly stirred mixture of 135 ml. of 18 N sulfuric acid[4] and 2500 ml. of warm water. The suspension was digested on the steam bath for a short time and the small excess of sulfuric acid was then quantitatively removed by the addition of the requisite amount of cold barium hydroxide solution. The barium sulfate was centrifuged and washed three times with hot water and the solution was concentrated *in vacuo* to 1 liter. It was then boiled with 10 gm. of norit, filtered, and further concentrated to 500 ml. Titration of an aliquot showed the presence of 145 gm. of isocitric acid instead of the theoretical 153 gm. Another aliquot was boiled with a slight excess of alkali and titrated with acid; there was no difference in acid content of the solution, showing that no significant lactonization had occurred up to this stage.

A portion of the solution that contained 107.3 gm. of isocitric acid was concentrated to a sirup which was heated in a boiling water bath for 10 minutes under atmospheric pressure. The vessel was then evacuated and

[3] Barium isocitrate has a negative temperature coefficient of solubility.
[4] A mixture of equal volumes of concentrated sulfuric acid and water.

heating continued while the sirup began to crystallize. This treatment was repeated several times. The mass was twice heated for 15 minutes with 250 ml. portions of toluene which was then distilled off *in vacuo*. The residue was transferred to a mortar with the aid of about 60 ml. of warm ethyl acetate and was warmed and ground until all had been converted to a granular solid. This was suspended in 300 ml. of toluene, heated to boiling, and chilled overnight. After being filtered, washed with toluene, and dried at 100°, the yellowish brown crude product weighed 96.6 gm., sintered at 142°, and melted at 152°.[5]

Fractionation of Diastereoisomers of Isocitric Lactone—A suspension of 106 gm. of crude lactone in 400 ml. of ethyl acetate was boiled gently for 30 minutes, chilled overnight, and filtered. The crystalline solid was washed with cold solvent and dried at 100°; it weighed 71.8 gm. and melted at 162–163°. The product was free from aconitic acid and the melting point was unchanged by recrystallization.

The mother liquor was concentrated *in vacuo* to 75 ml., heated to boiling, and 50 ml. of hot toluene were added. The crystals that separated after the solution had been chilled weighed 20.6 gm., and melted at 158°; nevertheless titration gave figures that corresponded to pure lactone. The material was recrystallized from a mixture of 100 ml. of ethyl acetate and 50 ml. of toluene, when 17.1 gm. of lactone of melting point 162–163° separated. The combined yield of lactone of this melting point was 88.9 gm. or 83.8 per cent of the crude material subjected to fractionation; analysis,[6] C 41.38, H 3.60; theory for $C_6H_6O_6$, C 41.39, H 3.47 per cent.

The combined mother liquors were evaporated to 15 ml. and an equal volume of ethyl acetate was added. Crystals began to separate after 24 hours, 8.4 gm. being finally collected. This material also gave titration figures that corresponded to those to be expected of pure isocitric lactone, but it melted at 118–120°; 7.0 gm. of the preparation were recrystallized from a mixture of 50 ml. of hot ethyl acetate and 15 ml. of toluene. The oil that began to separate was redissolved by warming the solution after

[5] The melting point of the crude lactone varied according to the quantity of ethyl acetate used in granulating the material, higher melting points being obtained if more of this solvent were employed. A series of nineteen preparations (mostly on a 2 gm. scale) gave melting points in the range 140–160°, although all were found to contain at least 98 per cent of lactone. The analysis was carried out upon 100 mg. samples, one of which was titrated with 0.1 N alkali to phenolphthalein, a second being treated with a known excess of about 10 ml. of 0.1 N alkali and boiled for 10 minutes before being titrated back with 0.1 N acid. The titration of the preparation described showed that 100 mg. contained 99.1 mg. of isocitric lactone. It gave a faint positive color test for aconitic acid with acetic anhydride and pyridine.

[6] Grateful acknowledgment is made to Mr. William Saschek of the Department of Biochemistry of the College of Physicians and Surgeons, Columbia University, New York, for this and other elementary analyses in this paper.

the addition of 3 ml. more of ethyl acetate. Crystals then formed slowly; the 5.2 gm. which finally accumulated sintered at 133° and melted at 136–138° to a turbid oil. The product was free from aconitic acid and by titration contained 99.9 per cent of lactone. From the mother liquors, a further 0.8 gm. of lactone was secured together with 0.037 gm. of crude aconitic acid, m.p. 193–194°, and 0.2 gm. of lactone contaminated with aconitic acid.

The fraction of melting point 136–138° was separated in a total yield of 6.8 per cent from the crude lactone. It was obviously not homogeneous but its high content of alloisocitric lactone was confirmed by the preparation of the p-bromophenacyl esters.

p-Bromophenacyl Esters of Isomeric Isocitric Lactones—A solution of 200 mg. of purified isocitric lactone of melting point 162–163° in 6 ml. of water was neutralized to phenolphthalein with 4.1 ml. of 0.56 N lithium hydroxide; 65 mg. of p-bromophenacyl bromide dissolved in 10 ml. of hot absolute alcohol were added and the mixture was boiled under a reflux condenser for 2 hours and then chilled. The product separated in fine needles that weighed 417 mg., a yield of 64 per cent, and melted at 189–.190°. The melting point was unchanged by repeated recrystallization. The ester is very slightly soluble in hot absolute alcohol; analysis, C 46.51, H 2.99, Br 27.97; theory for $C_{22}H_{16}O_8Br_2$, C 46.50, H 2.84, Br 28.13 per cent.

When treated in a similar manner, 100 mg. of the fraction of isocitric lactone that melted at 136–138° yielded 150 mg. of ester or 46 per cent of theory. The product was boiled with 25 ml. of absolute alcohol and filtered from an insoluble residue of 38 mg. This residue, when recrystallized from an ethyl acetate-alcohol mixture, melted at 189–190° and, when mixed with an equal quantity of the above derivative of the same melting point, showed no depression. The hot alcohol extract when cooled deposited 100 mg. of rosettes of fine needles which melted at 153–154° and were unchanged in melting point after four recrystallizations; analysis, C 46.42, H 2.96, Br 27.95 per cent. This was accordingly a pure specimen of the derivative of dl-alloisocitric lactone. A mixture in equal proportions of the purified specimens of the two isomeric derivatives gave at 162° a turbid oil which became clear at 170°.

Because of the low initial yield of these derivatives, it is impossible to estimate closely the relative proportions of isocitric and alloisocitric lactone in the fraction that melted at 136–138°. However, of the material actually brought to crystallization, it is clear that about two-thirds consisted of the derivative of the allo variety.

p-Chlorobenzyl Pseudothiouronium Salts—The salt was prepared essentially as described by Dewey and Sperry (12) from the lactone that melted

at 162–163°, the yield being 73 per cent. The product, after recrystallization from water, melted at 167–168°; analysis, C 45.89, H 4.34, Cl 12.05; theory for $C_{22}H_{24}O_6N_4S_2Cl_2$, C 45.91, H 4.20, Cl 12.32 per cent.

The sample rich in alloisocitric lactone gave, under the same circumstances, an oily product that was obtained crystalline with great difficulty and in poor yield. After recrystallization it also melted at 167–168°; a mixture with the other isomer melted at 160–161°. The derivative was unsatisfactory for identification purposes and was not analyzed.

p-Nitrobenzyl Pseudothiouronium Salts—p-Nitrobenzyl pseudothiouronium bromide was prepared according to the directions of Donleavy for the benzyl derivative, and the ester with isocitric lactone of melting point 162–163° was obtained, as described by Dewey and Sperry. The product separated in a yield of 72 per cent and was recrystallized from water. It melted at 153–155°; analysis, C 43.66, H 4.32; theory for $C_{22}H_{24}O_{10}N_6S_2$, C 44.29, H 4.05 per cent.

The alloisocitric lactone derivative was not obtained in satisfactorily homogeneous condition.

Toluidine Salts—To 1 gm. of isocitric lactone of melting point 162–163° in 40 ml. of dry ethyl acetate was added 0.7 gm. (1 equivalent = 0.62 gm.) of toluidine in 5 ml. of dry toluene. The salt began to separate within 2 minutes and 1.15 gm. were collected after the solution had been chilled overnight. The mother liquor yielded 0.33 gm. on concentration, a total of 90 per cent. After recrystallization from absolute alcohol, it sintered at 142° and decomposed at 146–148°; analysis, N 5.00; theory for $C_{13}H_{15}O_6N$, N 4.98 per cent.

The ditoluidine salt could be obtained either by recrystallizing the monotoluidine salt from ethyl acetate in the presence of an excess over a second equivalent of base. or from the lactone by employing approximately 3 equivalents. It separated in nearly quantitative yield as long narrow prisms. The best preparations decomposed near 155° but all were somewhat low in nitrogen (*e.g.* 6.85 instead of the theoretical 7.21 per cent) and it is doubtful that a homogeneous product was secured, the chief contaminant probably being the monosalt. The disalt could be recrystallized without decomposition only when excess of base was present.

The ditoluidine salt of alloisocitric lactone separated slowly and in a yield of only 57 per cent under the same conditions. It melted to a turbid oil at 133° and decomposed at 140°. This behavior was unchanged after recrystallization, but a mixture with the isomeric salt melted at 127° and decomposed at 131°. Since the product was not secured in pure form, no analysis was made.

Oxalosuccinic Ester—The procedures of Wislicenus (13) and of Blaise

and Gault (14) were followed with minor modifications, commercial sodium methylate being used as condensing reagent. The product, after being dried *in vacuo*, yielded 32.75 per cent of oxalic acid on hydrolysis in alkaline solution; theory for $C_{12}H_{18}O_7$, 32.84 per cent. In different preparations, the yield ranged from 80 to 87 per cent of crude ester, based on the sodium methylate used, and 72 to 78 per cent of ester purified through the potassium salt and corrected for the diethyl succinate recovered.

Triethyl Isocitrate by Method of Wislicenus and Nassauer—Repetition of the reduction of oxalosuccinic ester with sodium amalgam in aqueous suspension according to the conditions advocated by Wislicenus and Nassauer gave about 50 per cent of theory of an oil of which only about 70 per cent was triethyl isocitrate. The low boiling fraction (55–60° at 3 mm.) was rich in diethyl succinate (identified by means of the dihydrazide of melting point 164–165°). The aqueous solution, after extraction with ether, yielded an appreciable quantity of isocitric acid as the barium salt.

As the combined yield was still unsatisfactory, a study of the reduction reaction was undertaken. The most important modification developed was the use of alcohol and water mixtures rather than water as the reaction medium. This change increased the yield and eliminated the formation of significant quantities of diethyl succinate. However, the formation of isocitric acid subsequently isolated from the aqueous phase was again observed, which suggested that saponification of part of the ester may have taken place in the faintly acid solution (see (15)).

The conditions finally adopted were as follows: 25 gm. of oxalosuccinic ester were dissolved in a mixture of 125 ml. of alcohol and 225 ml. of water, and 3 per cent sodium amalgam (16) was added at the rate of about 45 gm. per hour with continuous mechanical shaking until 400 gm. had been used. The reaction was maintained at or near pH 5 (Congo paper) by the dropwise addition of 18 N sulfuric acid at 15 minute intervals. Shaking was then continued for an additional 6 hours. · The following morning the solution was adjusted to pH 5 and 10 gm. more of amalgam were added. After being shaken for 1 hour, the mixture was alkaline to Congo red and acid to litmus. During the entire process, from 12 to 14 ml. of sulfuric acid had been added. The suspension of sodium sulfate in the reaction flask was decanted from the mercury and washed on a funnel successively with cold water and with 50 per cent alcohol. The filtrate was concentrated *in vacuo* and, after the alcohol had been removed, was acidified with 2 ml. of 18 N sulfuric acid and extracted with ether. The extract gave a faintly positive test with ferric chloride for oxalosuccinic ester. This was removed by repeated treatment with a mixture of 20 per cent aqueous sodium sulfate solution and 1 N potassium carbonate in

the proportions of 3:2 and used in 25 ml. portions until the wash fluid was permanently alkaline. The ether extract was then washed with 20 per cent aqueous sodium sulfate until neutral and was dried with anhydrous sodium sulfate. Unchanged oxalosuccinic ester to the extent of 1.1 gm. was recovered from the potassium carbonate washings. The triethyl isocitrate that remained after distillation of the ether weighed 15.8 gm., a yield of 66 per cent of theory based on ester reduced.

The aqueous solution that remained after extraction of the ester was treated with 2.5 volumes of alcohol and chilled. The sodium sulfate that separated was removed and washed with dilute alcohol and the filtrate was concentrated in vacuo to 300 ml. To this were added 25 gm. of barium hydroxide octahydrate in 100 ml. of hot water and the mixture was heated on the steam bath for 2 hours. The barium salt was isolated and decomposed and, after concentration of the solution, acidity equivalent to 3.82 gm. of isocitric acid was found (equivalent to 5.5 gm. of triester), indicating that the total yield may have been as great as 89 per cent. After esterification, 4.9 gm. of triethyl isocitrate were recovered and added to the main fraction. Distillation of the 20.75 gm. of ester gave 2 drops at 55° and 4 mm. (presumably diethyl succinate) and 19.66 gm. at 143–144° at the same pressure. The distillation residue weighed 0.35 gm. Isocitric ester was thus recovered in a yield of 94.7 per cent from the crude material and the yield of distilled ester was 82.2 per cent of theory, based on the oxalosuccinic ester actually reduced. In the interests of brevity, this material will be referred to as WN ester.

Isocitric Lactone (Mixture of Isomers from WN Ester)—18.4 gm. of triethyl isocitrate were saponified with barium hydroxide and the free acid was concentrated to a sirup and heated in vacuo for several periods amounting in all to nearly 2 hours. The use of toluene to promote dehydration was found inadvisable, since it gave rise to darkening and strong color tests for aconitic acid were then obtained. In none of several experiments was crystallization of the lactone observed, as was invariably the case with material obtained by the Fittig method. The product was a pale yellow and highly viscous sirup and contained 12.9 gm. of isocitric acid isomers by titration after hydrolysis of the lactone ring; calculated 12.8 gm.

The sirup was dissolved in 35 ml. of hot ethyl acetate, filtered from a trace of barium sulfate, concentrated to a sirup, and held over drierite in vacuo at 50° for 6 hours, and then chilled. No crystals separated within 2 days. The mass (11.9 gm.) was taken up in 18 ml. of hot ethyl acetate and cooled; crystallization then slowly began and the solution was diluted with 25 ml. of warm toluene, and chilled. A crop weighing 5.38 gm. and melting at 148–149° was separated. On recrystallization from 10 ml.

of ethyl acetate and 5 ml. of toluene, this yielded 2.95 gm. of crystals of melting point 162–163° together with a second crop of 0.66 gm. of the same melting point. A mixture with the entirely similar product obtained by the Fittig method melted at the same temperature. A subsequent small crop melted at 148°. Further efforts to obtain crystalline material from the mother liquors failed in spite of additional dehydration operations. It could be concluded, however, that the preparation designated WN ester contained at least 33 per cent of an isomer of isocitric acid identical with the main product of the Fittig and Miller method.

Titration of the material in the combined mother liquors indicated that the dehydration operations had finally been carried too far, inasmuch as implausibly high figures for the content of lactone were secured. On the assumption that a small proportion of an isocitric anhydride had been inadvertently formed the material was saponified with barium hydroxide, recovered as acid, and the lactonization step was repeated; whereupon reasonable figures for the lactone content of the sirup were obtained. The product weighing 5.4 gm. was dissolved in 5 ml. of hot ethyl acetate. Solid material gradually separated and 2.34 gm. (20.7 per cent of the lactone fractionated) of a product that melted to a turbid oil at 134–137° were isolated; titration showed this to be 97 per cent pure lactone. Recrystallization gave 0.35 gm. of material of melting point 135–138°, which showed no depression when mixed with the product of the same melting point range obtained by the Fittig method and was found by titration to be 100 1 per cent lactone. The p-bromophenacyl ester melted at 157–159° but on recrystallization melted at 152–154°, and this melting point was not changed by four further recrystallizations. When mixed with an equal proportion of the purified derivative of alloisocitric lactone obtained by the Fittig method, there was no depression in melting point.

A second crop of 0.56 gm. of the lactone, secured from the mother liquors, gave a p-bromophenacyl ester which melted at 159–160°. On recrystallization this melted at 152–153° and was unchanged on further recrystallization.

It could be concluded that the WN ester contained an isomer of isocitric acid identical with that designated alloisocitric acid and obtained in small proportion by the Fittig method, and there was little reason to doubt that the proportion present was considerably in excess of the approximately 8 per cent actually isolated in impure form.

SUMMARY

The synthesis of isocitric acid by the hydrolysis of trichloromethyl-paraconic acid leads to a mixture of two diastereoisomeric forms in relative proportions of between 5:1 and 6:1, the racemic modification of the

natural optically active acid being the predominating component. This method, originally described by Fittig and Miller, has been modified in detail with improvement in yield.

The synthesis of isocitric acid by the reduction of oxalosuccinic ester and subsequent hydrolysis, as described by Wislicenus and Nassauer, leads to a mixture of isomers in more nearly equal proportions. The separation of this mixture is difficult and unsatisfactorily small yields of the lactone of the desired racemic acid were secured in pure form.

Alloisocitric acid was obtained in small yield from the reaction products of both methods but was not successfully purified as the lactone. Derivatives of this isomer were shown to differ from the corresponding derivatives of the racemic modification of the natural acid.

Several derivatives of both isocitric lactone and of alloisocitric lactone have been described. Of these, the p-bromophenacyl esters are useful both for characterization and for separation of the isomers. The neutral p-toluidine salt of isocitric lactone has potentialities for the large scale purification of this substance. Neither the trihydrazide of isocitric acid nor any of several pseudothiouronium salts of the lactones proved to be advantageous for characterization or for separation of the isomers.

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THE MAINTENANCE OF NITROGEN EQUILIBRIUM IN DOGS BY INTRAVENOUS ALIMENTATION WITH AN ACID HYDROLYSATE OF CASEIN FORTIFIED WITH TRYPTOPHANE

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Since McCoy, Meyer, and Rose's (1) discovery of threonine and its indispensability to the animal system, numerous studies in animals and in man have been reported on the administration of amino acids derived from various sources. Using a complete mixture of purified amino acids in a synthetic diet, Rose *et al.* (2, 3) have shown that it is possible to keep dogs and human beings in nitrogen equilibrium. Also Madden *et al.* (4) have obtained positive nitrogen balance with hypoproteinemic dogs injected with mixtures of the ten essential amino acids.

Amino acids are normally present in the blood stream. Therefore it is to be expected that they can be safely administered parenterally. In the normal animal amino acids are absorbed from the gastrointestinal tract. They are circulated in the blood stream and utilized in part for the synthesis of body proteins. A complete mixture of amino acids administered intravenously should thus be metabolized in the same manner. However, in malnutrition and starvation, when pathologic changes have occurred in the intestinal wall, the administration of amino acids or proteins may not be as effective orally as parenterally (5).

Recently investigators (6–15) have studied the parenteral administration of hydrolysates and mixtures of pure amino acids. Although beneficial results were clinically observed, many of the experiments were limited to short periods of time. This raises the question of retention of the nitrogen rather than utilization. Also most of the published work has not taken into account the excretion of nitrogen in the feces. Some investigators used the hydrolysate of proteins as a supplement to the diet. In these instances the rôle that such preparations play in the maintenance of nitrogen balance is open to question. Furthermore, in almost all of the above studies large amounts of nitrogen were administered.

The present investigation was undertaken to determine whether or not it is possible to maintain nitrogen balance in dogs over a sufficiently long period of time on known, low nitrogen intakes by (1) the oral administration of a complete mixture of amino acids produced by the acid hydrolysis

of casein, fortified with *dl*-tryptophane,[1] and (2) the parenteral adminis-
tration of such a preparation. The method for making a sterile, pyrogen-
free acid hydrolysate of casein has been described elsewhere (16) and the
quantitative estimation of its indispensable amino acids has been reported
by Block (17).

<center>EXPERIMENTAL</center>

The procedure employed was the same as that used by Rose *et al.*[2]
in their investigation of the utilization of mixtures of purified amino acids.
Three adult female dogs were used throughout the duration of the experi-
ment and were kept in individual metabolism cages. Each animal was
catheterized once daily and the 24 hour urine samples were diluted to a
standard volume and preserved with thymol in the refrigerator. The
feces were divided into 7 day periods by the use of carmine capsules and
were kept in acid alcohol. Total nitrogen of the urine and feces was
determined by the Kjeldahl procedure as modified by Scales and Harrison
(18). A methylene blue, methyl red indicator was used in the saturated
boric acid (19). Creatinine and creatine analyses were carried out by the
colorimetric methods of Folin (20) with Peters' (21) modification of time
factors. Consistent daily creatinine excretion was used to indicate that
a complete 24 hour urine sample had been obtained.

The diet presented in Table I is an adaptation of Cowgill's (24). The
protein fraction of the diet was omitted and the amino acids were fed
either by stomach tube or by vein. Nitrogen of the food other than that
furnished by the amino acids amounted to 60 mg. per 100 gm. of diet, of
which approximately 40 mg. were contained in the vitamins. The 20 mg.
of nitrogen of unknown origin represent less than 2 per cent of the total
nitrogen intake even though the total nitrogen intake is very low. Thus
the nitrogen in the diet is substantially derived from the acid hydrolysate
of casein fortified with tryptophane.

At the beginning of each experiment the animal was fed a 6 per cent
casein diet supplemented with methionine[3] for at least 2 weeks. The
casein and methionine were then replaced by the acid hydrolysate fortified
with tryptophane for 1 or more weeks. Subsequently this preparation
was injected either intravenously or intraperitoneally for 7 day periods
respectively. The remaining portions of the diet were fed orally. Fol-
lowing the period of parenteral administration, the hydrolysate was again
fed by stomach tube. Throughout the experiment the dog was given
approximately 80 calories per kilo of body weight.

Results

Since all the animals reacted very similarly, the complete data for only one dog are given in Table II.   The nitrogen balance data are summarized in Figs. 1 to 3.

It can be seen from the data given in Table II that the creatinine excretion is constant.   This has been noted in all normal adult dogs studied. The creatine excretion, however, is quite variable.   The small amount of nitrogen in the feces represents a rather large percentage of the total nitrogen excreted (15 to 20 per cent).   The fecal nitrogen is probably close to an irreducible minimum.   When no appreciable nitrogen is fed,

TABLE I

Composition of Diet .

	calories	gm.	Vitamin supplement per 100 gm.	γ
Sucrose.................	160	40.0	Riboflavin..............	600
Dextrin................	86	21.50	Thiamine HCl..........	600
Methylcellulose.........		2.50	Pyridoxine HCl.........	600
Salt mixture*..........		4.00	Calcium d-pantothenate.	600
Lard.....	177.75	19.75		mg.
Cod liver oil...........	27.00	3.00	Nicotinic acid.........	6
Wheat germ oil........	2.25	0.25	Choline chloride........	300
Liver extract†.........		2.00		
		93.00		
Casein hydrolysate fortified with tryptophane (fed separately)......	28.00	7.00		
	481.00	100.00		

Approximately 15.5 gm., per kilo of body weight of the dog, of the protein-free diet were fed.

* Jones and Foster (22).

† Conger and Elvehjem (23).   Fullers' earth treatment omitted.

the feces obtained still contain approximately the same amount of nitrogen. The largest animal, receiving more nitrogen, excreted a greater amount in the feces, but a slightly lower proportion of the total nitrogen eliminated (14 per cent).   In no case did the nitrogen in the feces fall below 12 per cent of the total nitrogen output.

The data presented in Table II show that Dog 2 retained nearly 20 per cent of the nitrogen fed, whereas the others kept 10 to 15 per cent.   During the week of intraperitoneal injections of Dog 2, only 1.7 per cent of the nitrogen administered was held (Fig. 1).   However, during all the other parenteral feedings, at least 15 per cent of the nitrogen was not excreted,

which is approximately the same as when the amino acids are fed orally. As was anticipated, the dogs slowly gained weight. While there are days
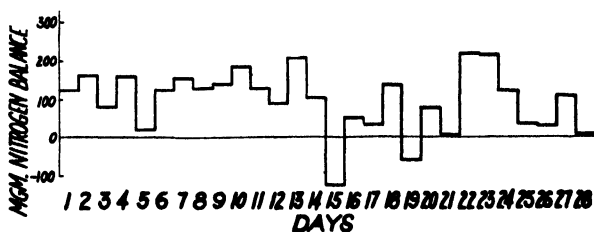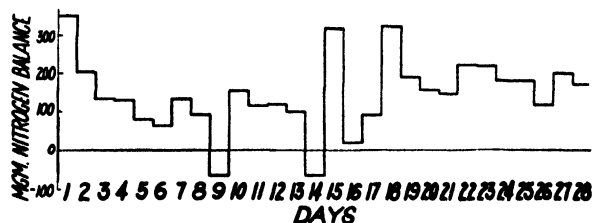

FIG. 1. Dog 2, 4.9 kilos. Nitrogen balance with an acid hydrolysate of casein fortified with tryptophane as the sole source of amino acids. Nitrogen intake 1125 mg. per day $\approx$ 230 mg. of nitrogen per kilo of body weight. Days 1 to 14, oral administration; days 15 to 21, intraperitoneal administration; days 22 to 28, oral administration.

FIG. 2. Dog 3, 16.3 kilos. Nitrogen balance with an acid hydrolysate of casein fortified with tryptophane as the sole source of amino acids. Nitrogen intake 3850 mg. per day $\approx$ 236 mg. of nitrogen per kilo of body weight. Days 1 to 21, oral administration; days 22 to 28, intravenous administration; days 29 to 35, oral administration.

FIG. 3. Dog 1, 5.6 kilos. Nitrogen balance with an acid hydrolysate of casein fortified with tryptophane as the sole source of amino acids. Nitrogen intake 1200 mg. per day $\approx$ 214 mg. of nitrogen per kilo of body weight. Days 1 to 14, oral administration; days 15 to 21, intravenous administration; days 22 to 28, oral administration.

of negative nitrogen balance, these are the usual variations one obtains with normal adult animals, and the over-all picture is one of good positive

balance.   Figs. 1 to 3 show that good positive nitrogen balance has been maintained in every dog used, with the acid hydrolysate of casein, to which

TABLE II

Nitrogen Balance with Casein Hydrolysate Fortified with Tryptophane As Sole Source of Amino Acids

Dog 2.

Amino acids fed (total N intake, 1000 mg.)	Date	Body weight	Urine N	Creatinine N	Creatine N	Fecal N*	Total N output	N balance
		kg.	mg.	mg.	mg.	mg.	mg.	mg.
Orally	Mar. 14	5.3	676	32	16	142	818	+182
	" 15	5.3	705	32	14	142	847	+153
	" 16	5.4	648	32	. 6	142	790	+210
	" 17	5.4	655	31	7	142	797	+203
	" 18	5.4	675	32	3	142	817	+183
	" 19	5.4	633	31	3	142	775	+225
	" 20	5.4	728	32	3	142	870	+130
Total..............			4720	222	52	994	5714	+1286
Average...........		5.4	674	32	7	· 142	816	+184
Intravenously	Mar. 21	5.4	· 766	32	10	125	891	+109
	" 22	5.4	870	31	. 14	125	995	+5
	" 23	5.3	583	34	3	125	708	+292
	" 24	5.4	615	31	3	125	740	+260
	" 25	5.3	644	32	3	125	769	+231
	" 26	5.3	635	34	3	125	760	+240
	" 27	5.3	· 676	33	2	125	801	+199
Total..............			4789	227	38	875	5664	+1336
Average.....		5.3	684	32	5	125	809	+191
Orally	Mar. 28	5.3	512	33	2	158	670	+330
	" 29	5.4	571	33	2	158	729	+271
	" 30	5.4	631	33	3	158	789	+211
	" 31	5.5	823	35	2	158	981	+19
	Apr. 1	5.4	615	33	4	158	773	+227
	" 2	5.4	648	34	2	158	806	+194
	" 3	5.4	666	31	5	158	824	+176
Total..............			4466	232	20	1106	5572	+1428
Average...........		5.4	638	33	3	158	796	+204

* The total fecal nitrogen was determined once for each period; thus the daily figures are averages, which accounts for their uniformity.

1 per cent of tryptophane has been added, as the sole source of amino acids.   The nitrogen intakes are near minimal and were chosen because of

the practical importance of maintaining balance with the least amount of fluid administration. It is possible to obtain much larger nitrogen retention for short periods at high nitrogen intakes, but in view of the clinical conditions which must normally be met low levels of nitrogen intake were used.

SUMMARY

Three different dogs have been maintained for 21 to 35 days in positive nitrogen balance by the administration of purified diets wherein practically the sole source of nitrogen was derived from an acid hydrolysate of casein fortified with tryptophane. The preparation was given both orally and parenterally to supply a nitrogen intake of approximately 200 mg. per kilo of body weight.

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THE ACTIVITY OF *dl*-OXYBIOTIN FOR THE RAT*

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The total synthesis of a compound, differing structurally from biotin only in the replacement of the sulfur by oxygen, has been described (2). This oxygen analogue of biotin has been named oxybiotin, both because of its structural relationship to biotin and its ability to replace this substance as an essential metabolite for various species of microorganisms and higher animals. The present paper is one of a series relating to the biological activity of *dl*-oxybiotin (1, 3–6) and is concerned with the relative effectiveness of *d*-biotin and *dl*-oxybiotin in the cure of egg white injury in the rat.

EXPERIMENTAL

Two independent experiments, designated as Series I and II, were conducted. Each series consisted of thirty male, 26 day-old, albino rats (Sprague-Dawley), weighing 40 to 50 gm. The animals were housed in individual cages with wide mesh screen bottoms. Biotin deficiency was induced by feeding *ad libitum* purified diets containing commercial dried egg white (7). The composition of the diets employed in Series I and II is given in Table I. The B complex vitamins[1] were fed daily in supplement dishes at the following levels: thiamine hydrochloride 30 γ, pyridoxine hydrochloride 30 γ, riboflavin 30 γ, nicotinic acid 250 γ, calcium pantothenate 200 γ, choline chloride 10 mg., and *i*-inositol 3 mg. 1 drop of haliver oil containing 1.3 mg. of added *dl*-α-tocopherol acetate and 0.8 mg. of added 2-methyl-1,4-naphthoquinone was given weekly to each rat.

Growth of the animals ceased after 7 weeks in Series I, and 5 weeks in Series II. A weight plateau was maintained during the succeeding 2 weeks in each series. The administration of the test substances was then begun. At this time, the average weight of the rats in Series I was 150 gm., while that in Series II was 148 gm. Marked symptoms of biotin deficiency characterized by cheilosis, spectacled eye, dermatitis, and alopecia were evident.

For the therapeutic studies, the animals in each series were divided into six groups (five rats per group) of similar average weight and severity of the skin lesions. *d*-Biotin, *dl*-oxybiotin, or physiological saline was administered

[1] We are indebted to Merck and Company, Inc., for the synthetic vitamins.

TABLE I

Composition of Diets (Gm. per 100 Gm. of Diet)

Component	Series I	Series II
Sucrose	76	74
"Vitamin-free" casein (Labco)	8	
Dried egg white*	10	20
Salts 4 (8)	4	4
Corn oil	2	2

* The batches of egg white employed in Series I and II were purchased from two commercial sources.

TABLE II

Comparison of Activities of d-Biotin and dl-Oxybiotin

Group*	14 day curative period		28-day curative period	
	Average change in weight†	Per cent activity of dl-oxybiotin (d-biotin = 100)‡	Average change in weight†	Per cent activity of dl-oxybiotin (d-biotin = 100)‡
Series I				
	gm.		gm.	
Negative control	−3			
0.1 γ of d-biotin	+18		+35	
0.2 " " "	+32		+60	
0.4 " " " §	+43		+77	
0.2 " " dl oxybiotin	−3			
0.4 " " "	−2			
2.0 " " "	+21	6.0	+37	5.3
Series II				
0.1 γ of d-biotin . .	+19		+34	
0.2 " " "	+32		+50	
1.0 " " "	+58		+98	
2.0 " " dl-oxybiotin	+17	4 5	+25	3.8
4.0 " " "	+25	3.7	+44	4.1
20.0 " " "	+44	2.9	+72	2.9

* The dosages are expressed in terms of the daily supplementation.

† Total weight changes over the designated time interval are given.

‡ The potency of oxybiotin in terms of biotin activity was determined from calibration curves made by plotting growth responses to d-biotin at both the 14 and 28 day periods against the graded amounts of d-biotin. All calculations were made on a weight basis.

§ These animals served previously as the negative controls.

daily by subcutaneous injection, as shown in Table II. Biotin and oxybiotin were prepared in physiological saline at concentrations furnishing the

requisite daily supplementation per cc. of solution.   1 cc. of physiological saline was administered daily to the negative control group of Series I. The animals continued to receive the egg white-containing diets during the curative assay period.   In Series I, the negative control group and the groups receiving 0.2 γ and 0.4 γ of dl-oxybiotin were given treatment for 2 weeks.   In all other groups of both series the test substances were administered for 28 days.

Results

The growth responses to d-biotin and dl-oxybiotin are summarized in Table II.   With growth stimulation as the criterion, the activities of dl-oxybiotin compared to that of d-biotin and expressed on a percentage basis varied from 2.9 to 6.0 per cent.   The average activity was 4 per cent. Approximately the same results were obtained in both the 14 day and 28 day assays.   The lowest activity ratio was obtained at the highest level of oxybiotin (20 γ) and was partially due to the lag in growth response to this level of oxybiotin noted during the early phase of the curative test period. A similar lag was not observed when 1.0 γ of d-biotin was administered.

The rate and extent of cure of the skin lesions were proportional to the dosages of biotin and oxybiotin.   Complete cure was effected in 28 days by the daily administration of 1.0 γ of d-biotin or 20 γ of dl-oxybiotin.   The relative activities of biotin and oxybiotin with respect to cure of the lesions were comparable to those based upon the growth responses.

DISCUSSION

The experiments recorded in this paper demonstrate that dl-oxybiotin is a therapeutic agent for egg white injury in the rat.   With regard to its ability to promote growth, dl-oxybiotin is 4 per cent as effective as d-biotin. Similarly, dl-oxybiotin is less active than d-biotin in the cure of the skin lesions.   However, it should be noted that dl-oxybiotin is capable of completely alleviating the skin symptoms.   The few cases of spastic paralysis of the hind legs observed in these studies were also completely cured by dl-oxybiotin.   Thus, the curative action of a sufficient dosage of dl-oxybiotin upon the skin lesions and the spasticity of the hind legs is indistinguishable from that of d-biotin.   Comparable activity in the rat for a compound apparently similar to dl-oxybiotin has been observed by Rubin et al. (9). It is of interest that dl-oxybiotin is 17 per cent as active as d-biotin for the chick (6).   For various species of microorganisms, the activities of dl-oxybiotin range from 15 to 50 per cent that of d-biotin (4, 9).

In all of the studies to date, an optically inactive form of oxybiotin has been employed.   It appears likely, in analogy with dl-biotin (10), that only one of the enantiomorphs of dl-oxybiotin is biologically active.   If such is

the case, the activities of the biologically active enantiomorph would be twice the values reported for *dl*-oxybiotin.

Of the various derivatives of biotin tested for biological activity in higher animals (11–13), oxybiotin is the only compound possessing activity comparable to that of biotin. Thus *dl*-oxybiotin affords a unique example of the ability to effect a substitution in the ring structure of a vitamin and still retain considerable biological activity.

The question of the mechanism of action of oxybiotin presents itself. Can oxybiotin replace biotin in the metabolism of the rat or does oxybiotin function merely as a precursor substance for biotin? At the present, this question must remain unanswered. Balance studies now in progress may serve to clarify this problem.

SUMMARY

The activity of *dl*-oxybiotin in the cure of egg white injury in the rat has been determined. With growth response as a criterion in either a 14 or 28 day curative assay procedure, *dl*-oxybiotin was found to possess 4 per cent of the activity of *d*-biotin. Complete cure of the skin lesions was effected by the daily administration of 20 γ of *dl*-oxybiotin for 28 days.

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THE POSITION OF THE HIGHER FATTY ALDEHYDES IN FATTY ACID METABOLISM OF RAT MUSCLE*

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Two sets of experiments were designed to determine the rôle of the higher fatty aldehydes in the metabolism of their closest structural relatives, the fatty acids. Adult male rats on a low fat diet (*a*) were maintained on a heavy water régime or (*b*) were fed fat containing deuterium. In both groups of experiments the rats were killed after different time intervals and the deuterium content of the higher fatty aldehydes and of the fatty acids in different lipid fractions of the muscle was determined.

The experiments were carried out on skinned, eviscerated, and decapitated carcass, since the lipid fraction of muscle contains, next to brain, the highest concentration of the higher fatty aldehydes of all organs studied. A previous investigation (1) made available a relatively simple technique for isolation of the higher fatty aldehydes as *p*-carboxyphenylhydrazones from small amounts of tissue. This procedure has now been modified for routine use.

It has been found that large amounts of lipids may retain the acidic hydrazones in the organic solvent. By a short alkaline hydrolysis of the lipid emulsion prior to the liberation of the aldehydes by acid enough of the lipids is saponified to make the isolation of the hydrazones possible in practically every experiment. This modification has not been successful in the routine isolation of the aldehydes from brain tissue.

In the isolation procedure the higher fatty aldehydes are exposed to action of strong alkali and acid. It appeared unlikely from data on fatty acids (2) that such a treatment would labilize any deuterium bound to the carbon chain. Since aldehydes in their naturally occurring form (acetal phosphatides) containing deuterium are not available, the uptake of deuterium by aldehydes was studied on a lipid emulsion obtained from rat muscle and treated with alkali and acid in the presence of heavy water. It was found that approximately 1 per cent of the hydrogen atoms of the fatty aldehydes was exchanged during the isolation procedure. The correction resulting from an exchange of this magnitude is too small to affect the interpretation of the results.

Administration of Heavy Water—If the higher fatty aldehydes are intermediates in the synthesis of the fatty acids, representing *e.g.* the last stage of an aldol condensation (3), they should contain a higher concentration of deuterium than the fatty acids if the synthetic processes take place in animals, the body water of which is enriched with heavy water. It is immaterial whether muscle or some other organ is the site of synthesis because the synthesized aldehydes would be transferred from the organ of synthesis to the muscle and the final oxidation to the fatty acid would take place in this organ. Since the conversion from aldehyde to acid involves only the terminal aldehyde group, it may be assumed that no metabolic labilization and

TABLE I

Deuterium in Higher Fatty Aldehydes and Fatty Acids of Muscle of Rats Given Heavy Water

Experiment No.	No. of rats	Duration of experiment	D₂O in body water	Deuterium in				Hydrogen from body fluids in			
				Higher fatty aldehydes	Fatty acids			Higher fatty aldehydes	Fatty acids		
					Total	Phosphatides	Neutral fat		Total	Phosphatides	Neutral fat
		days	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1	4	0.7	5.0	0.03				0.6			
	2		5.0			0.15	0.13			3.0	2.6
2	10	6	0.83	0.066	0.12*			8.0	14.5		
3	8	6	1.54	0.11	0.13†			7.0	8.5		
	2		1.54			0.13‡					8.6

* Iodine No. 79; unsaturated fatty acids 0.08 atom per cent deuterium.

† Iodine No. 83; unsaturated fatty acids 0.08 atom per cent deuterium, saturated fatty acids 0.17 atom per cent deuterium.

‡ Iodine No. 134.

loss of deuterium would occur during this step. In Table I are recorded the results of three experiments, one of 18 hours and two of 6 days' duration. In all three experiments the higher fatty aldehydes contained less deuterium than either the total fatty acids or the fatty acids isolated from phosphatides or from neutral fat. This finding contraindicates any simple relationship between the synthesis of the aldehydes and of the fatty acids, particularly since the aldehydes seem to correspond to the saturated fatty acids of chain length C_{16} or C_{18}. In such experiments the saturated fatty acids will contain a higher concentration of deuterium than that of the total fatty acids of any lipid fraction (4, 5). The unsaturated fatty acids in Experiments 2 and 3 contained a deuterium concentration corresponding to 64 per cent of that found in the total fatty acids. The saturated fatty acids, therefore,

contained a deuterium concentration considerably higher than that of the total fatty acids and probably about twice that found in the unsaturated fatty acids.   The difference in deuterium concentration between the aldehydes and the saturated fatty acids is therefore still more marked.   The close proximity of the deuterium concentrations of unsaturated fatty acids and aldehydes appears at this stage of the investigation to be purely accidental, since the unsaturated fatty acids include essential fatty acids which contain very little deuterium (4, 5).

The experiments with heavy water administration appear to yield information on the metabolism and the turnover of the fatty acids of muscles hitherto not obtained with this technique.   The phosphatide fatty acids contain a slightly higher deuterium concentration than the fatty acids derived from neutral fat, as may be seen from the direct determinations of Experiment 1.   This finding is in keeping with the view that the phosphatides are active intermediates in fatty acid metabolism (6, 7).   The higher proportion of unsaturated fatty acids in the phosphatides (compare iodine numbers in Experiment 3) than in the total fat, and therefore in the neutral fat fraction, makes the difference in deuterium concentration in the saturated fatty acids still larger than that expressed by the analytical data.

In previous experiments carried out under the same conditions it was found that the liver fatty acids contain a higher deuterium concentration than the fatty acids of intestine, carcass, or brain (8).   The values were in agreement with the view that the liver fatty acids of rats (6) and mice (9) are half replaced in less than 1 day.   In experiments of 6 days' duration the deuterium concentration in the carcass fatty acids, which comprised those from skin, muscle, and skeleton, amounted to about 30 per cent of that found in the liver.   The percentage of hydrogen derived from body water in the fatty acids of muscle, as reported in this paper, is similar to that previously found for carcass.   This result suggests that the rate of replacement and turnover of newly synthesized fatty acids is about the same in muscle as in the eviscerated and decapitated carcass (8), and is considerably slower in muscle than in liver if it is assumed that there is no larger influx of fatty acids from the stores into the muscle than into the liver.

In the previous experiments (8) the surprising observation was made that the unsaponifiable material from decapitated and eviscerated carcass contained a percentage of hydrogen derived from body water higher than that of the unsaponifiable constituents of any other organ studied (liver, intestine, brain) and also higher than that derived from body water in the fatty acids of the carcass.   In the two experiments of 6 days' duration reported in Table I the unsaponifiable material from the muscle was prepared and analyzed for its deuterium concentration.   The percentages of hydrogen derived from body fluids were 12 per cent (0.10 atom per cent deuterium)

and 6 per cent (0.09 atom per cent deuterium) respectively. These values are approximately one-half of those of the carcass and are in the range of the figures found previously for the unsaponifiable lipids of liver and intestine. The high metabolic activity of the unsaponifiable material of the carcass appears to be restricted to the skin, since it cannot be accounted for by muscle.

Administration of Fat Containing Deuterium—The experiments in which heavy water was administered to rats show that the higher fatty aldehydes are not involved in the over-all synthesis or transport of fatty acids and are in accord with the possibility that fatty acids and aldehydes are synthesized by different pathways in the animal organism. A metabolic relationship between higher fatty aldehydes and fatty acids is, on the other hand,

TABLE II

Distribution of Deuterium in Higher Fatty Aldehydes and Fatty Acids of Muscle of Rats after Administration of Fat containing Deuterium

Experiment No.	No. of rats	Duration of experiment	Deuterium in				Administered fatty acids in			
			Higher fatty aldehydes	Fatty acids			Higher fatty aldehydes	Fatty acids		
				Saturated (total)	Phosphatides	Neutral fat		Saturated (total)	Phosphatides	Neutral fat
		hrs.	*atom per cent*	*atom per cent*	*atom per cent*	*atom per cent*	*per cent*	*per cent*	*per cent*	*per cent*
1*	4	8	0.013				0.23			
	2				0.09	0.06			1.6	1.1
2†	6	16	0.022	0.06			0.34	0.9		

* 1.5 ml. of linseed oil (fatty acids 5.7 atom per cent deuterium).

† 0.7 to 1.0 ml. of linseed oil (fatty acids 6.45 atom per cent deuterium).

indicated by the experiments in which fat containing deuterium was administered to rats (Table II). In both experiments the higher fatty aldehydes contained a concentration of deuterium which, though small, was appreciable when compared with that found in the fatty acids and higher than that present in the body water (less than 0.01 atom per cent). In the experiment of 8 hours' duration the amount of fatty acids derived from the administered fat and converted into aldehydes amounted to 15 to 20 per cent of the concentration found in the fatty acid fractions. In the experiment of 16 hours' duration this value amounted to 40 per cent on the basis of the deuterium concentration determined in the saturated fatty acids. These findings suggest the ability of the animal body to reduce the fatty acids to the corresponding aldehydes. Whether the aldehydes are intermediates in the metabolically reversible conversion of fatty alcohols to fatty acids, as discussed previously (10, 11), is under investigation at present.

The results obtained in both types of experiments show that the higher fatty aldehydes are not main intermediates in the synthesis or transport of fatty acids, but take part in some special aspect of fatty acid metabolism.

Approximately the same percentage of administered fatty acids was present in the muscle fatty acids as was found previously in experiments of the same type (12) in the fatty acids of the eviscerated and decapitated carcass. The results suggest that the main source of fatty acids in muscle, as in eviscerated carcass, is not synthesis of fatty acids in the organ itself but transfer from other organs to the muscle.

In the experiments in which labeled fat was given to the animals the phosphatides and neutral fat of the liver were separated. 15 per cent of phosphatide fatty acids (0.87 atom per cent deuterium) in the 8 hour experiment was derived from the administered fat; i.e., 10 times more than was found in phosphatides of the muscle. In the 16 hour experiment 8.4 per cent (0.54 atom per cent) of the phosphatide fatty acids and 4.2 per cent (0.26 atom per cent) of the neutral fat fatty acids were derived from the administered fat; the concentrations are at least 10 times those found in the total fatty acids of the muscle. The finding of a higher percentage of administered fatty acids in the phosphatides than in the neutral fat confirms the high metabolic activity of the phosphatides (6).

EXPERIMENTAL

Preparation of Labeled Fat—The fat was prepared by hydrogenating linseed oil with deuterium in the apparatus of Rittenberg and Schoenheimer (13). Two preparations were used containing 5.7 and 6.45 atom per cent deuterium respectively in the fatty acids.

Animal Experiments—In each experiment six to ten male rats weighing 180 to 220 gm. were kept for at least 8 days on a low fat diet (12). The administration of the labeled fat or the deuterium oxide was not started until the animals had overcome the initial drop in weight. Enough 99.9 per cent deuterium oxide (Experiment 1, Table I) or water containing 50 per cent deuterium oxide (Experiments 2 and 3, Table I) to bring the body fluids to the calculated concentration of 5 or 1.5 atom per cent respectively was injected into the animals. The drinking water contained 12.5 or 2.5 atom per cent deuterium respectively. The labeled fat was administered by stomach tube.

The animals were killed by decapitation and muscles together with the bones were minced in a meat grinder. The muscles were not removed from the bones, in order to obtain a maximal amount of muscle and to shorten the time interval between death of the animal and extraction of lipids. Therefore, all our lipid fractions are contaminated with lipid material originating from bone marrow.

Isolation of Higher Fatty Aldehydes as p-Carboxyphenylhydrazones—The procedure has already been published in detail (1). Two modifications were introduced: (*a*) alkaline hydrolysis of the lipid emulsion and (*b*) addition of stearic acid at the proper step to avoid the contamination of the aldehydes with labeled fatty acids.

The muscle hash from four to ten rats is extracted exhaustively (seven times) with boiling 95 per cent ethanol. After removal of the alcohol *in vacuo* the remaining aqueous emulsion is made alkaline by the addition of 10 ml. of 30 per cent KOH per 100 ml. and is kept at 100–105° for 1 hour. A hydrolysis with alkali and of shorter duration was used by Feulgen and Bersin (3) in the preparation of acetal phosphatides.

When cold, the emulsion is acidified with 2 N sulfuric acid with Congo red as an indicator. For the liberation of the aldehydes the mixture is kept at 37° for 4 hours. The emulsion is distributed between ether and a 4 per cent solution of potassium carbonate and the ether solution is repeatedly extracted with potassium carbonate solution to remove all the free fatty acids. The extracted fatty acids represent part of the total fatty acids (Fraction A). When the carbonate solution shows only a faint cloudiness after acidification 1 gm. of stearic acid is dissolved in the ether solution and removed by extraction with carbonate. By this "washing out procedure" the last traces of labeled fatty acids are removed and diluted down to an insignificant deuterium concentration. The carbonate extract containing mainly the added stearic acid is discarded. The ethereal solution is washed with water, dried over sodium sulfate, and the ether removed. The coupling of the free aldehyde with p-carboxyphenylhydrazine in alcoholic solution is carried out as described previously (1). The alcoholic solution is distributed between ether and a 4 per cent solution of potassium carbonate; the acid hydrazone is extracted from the ethereal solution with the carbonate solution and is isolated and recrystallized as described. The ethereal solution contains the lipids not saponified by the alkaline treatment and the unsaponifiable material and represents Fraction B of the total fatty acids.

The melting points of the p-carboxyphenylhydrazones varied from 88–96° (Fisher-Johns melting point apparatus) and the analyses gave the following values: found, C 74.2 to 74.5, H 10.2 to 10.5;[1] calculated for $C_{23}H_{38}O_2N_2$, C 73.7, H 10.2; for $C_{25}H_{42}O_2N_2$, C 74.3, H 10.5.

Exchange of Hydrogen of Higher Fatty Aldehydes during Isolation Procedure—To 144 ml. of lipid emulsion prepared from the muscles of six rats, 16 ml. of 99.9 atom per cent heavy water and 5.8 gm. of KOH were added. The mixture was kept at 105° for 1 hour. When cold, the solution was acidified with approximately 60 ml. of 2 N H_2SO_4 and the isolation of the aldehydes was carried out as described. The p-carboxyphenylhydrazone

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[1] We are indebted to Mr. W. Saschek for the analyses.

contained 0.1 atom per cent deuterium. In another experiment the lipid emulsion during saponification contained 5 per cent of heavy water. 0.03 atom per cent deuterium was found in the hydrazone.

The experiments show that about 1 per cent of the hydrogen atoms of the aldehydes is exchanged during the isolation procedure.

Separation of Lipids; Total Fatty Acids—The fatty acids contained in the potassium carbonate solution (Fraction A) were extracted with ether after acidification and combined with Fraction B. The ether was removed and the residue was saponified by refluxing with alcoholic potassium hydroxide. The alcoholic solution was distributed between petroleum ether and water; fatty acids and unsaponifiable lipids were separated and prepared for analysis in the usual manner (12).

Phosphatides and Neutral Fat—Phosphatides and neutral fat were separated by the methods of Bloor (14) and Sinclair (15) from the muscle hash of two rats. The phosphatides were twice precipitated with acetone with the addition of an alcoholic solution of magnesium chloride. Phosphatides and neutral fat present in the supernatant liquid from the acetone precipitation were saponified with alcoholic potassium hydroxide and the fatty acids of both fractions isolated. For the separation of phosphatides and neutral fat from liver the organs of all animals in the experiment were used.

In the separation of saturated and unsaturated fatty acids the procedure of Twichell (16) was followed. The iodine numbers were determined by the method of Rosenmund and Kuhnhenn, as modified by Yasuda (17).

Determination of Deuterium—The deuterium content of the different fatty acid fractions was determined by the method of Keston, Rittenberg, and Schoenheimer (18). The deuterium content of the p-carboxyphenylhydrazones of the higher fatty aldehydes was determined by an unpublished micromethod developed by Rittenberg. We are highly indebted to Dr. Rittenberg and Mr. Sucher for the determinations.

SUMMARY

A modification of the previously reported procedure for isolation of the higher fatty aldehydes as p-carboxyphenylhydrazones is described. By this technique it is possible to isolate routinely the aldehydes from the muscles of a small number of rats.

The deuterium concentration of the higher fatty aldehydes, fatty acids of different lipid fractions, and unsaponifiable material of muscle of rats was determined after periods of 18 hours and 6 days respectively, during which the body fluids were enriched with deuterium through the administration of heavy water. In a second set of experiments the deuterium concentration was determined in the same components of the muscle lipids 8 and 16 hours after the administration of a single dose of fat labeled with deuterium.

In both types of experiments the higher fatty aldehydes contained appreciable amounts of deuterium, but invariably less than was present in the fatty acid fractions. This result indicates that the higher fatty aldehydes are not involved in the over-all synthesis or transport of fatty acids but are concerned with some special aspect of fatty acid metabolism. In both sets of experiments the rate of turnover of the muscle fatty acids corresponded roughly to that previously found for the fatty acids of the decapitated and eviscerated carcass.

The unsaponifiable lipids isolated from muscle of rats, the body water of which was enriched with heavy water, contained much less deuterium than those isolated from carcass previously under the same experimental conditions. The high metabolic activity of the unsaponifiable material of carcass appears to be restricted to the skin, since it cannot be accounted for by muscle.

The fatty acid fraction of the phosphatides of muscle and of liver showed a higher deuterium concentration than the fatty acids of neutral fat of the same organ.

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THE NITROGEN METABOLISM OF RAT TISSUE SLICES UNDER VARIOUS CONDITIONS

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When tissues are ground up and incubated aseptically, autolysis occurs and the proteolytic enzymes in the cell are considered responsible. It is assumed that after the death of an animal and while the cell structures are still intact similar autolytic action occurs. It is not, however, known whether autolysis begins immediately after death with the establishment of anaerobic conditions. It is possible that other changes in nitrogen metabolism and distribution precede autolysis. In order to investigate this possibility the changes in nitrogen metabolism of tissue slices during 3 or 4 hours post mortem were studied under various conditions and, in particular, the effects of anaerobiosis. It soon became evident that some of the changes observed under the conditions of the experiments could not be attributed to the result of autolytic action.

EXPERIMENTAL

200 gm. rats were killed by decapitation. The tissues were immediately removed and sliced as rapidly as possible in Krebs' bicarbonate solution. Before transfer into the experimental vessels the slices were washed in fresh solution and blotted on filter paper before weighing. 300 mg. of tissue (wet weight) were placed in each 50 cc. Erlenmeyer flask containing 4.0 cc. of solution which was incubated at 37° with either 95 per cent O_2 and 5 per cent CO_2, or 95 per cent N_2 and 5 per cent CO_2. The anaerobic condition produced by the latter mixture was sufficient to prevent the oxidation of p-phenylenediamine; *i.e.*, the cytochrome oxidase system was completely inactive. The procedure for analysis was as follows. An aliquot was removed for the determination of total nitrogen by the micro-Kjeldahl technique. To the remainder, including the slices, 1.0 cc. of 20 per cent trichloroacetic acid was added. The solution with the precipitated protein was poured off from the slices and centrifuged. An aliquot of the supernatant fluid was analyzed for non-protein nitrogen by micro-Kjeldahl, and another (after autoclaving) for ammonia nitrogen by vacuum distillation for 10 minutes from 20 per cent sodium hydroxide at 60°. The values from these procedures were obtained by nesslerization and the estimation of the color in the Evelyn colorimeter. Finally, an

aliquot was used for the determination of amino nitrogen by the Van Slyke nitrous acid method. Before this could be done, however, it was necessary to autoclave the solutions for 15 minutes at 20 pounds because, aerobically, liver and to some extent kidney slices produce acetoacetic acid, which liberates nitrogen from nitrous acid. The autoclaving in acid destroys the acetoacetic acid and does not apparently hydrolyze peptide bonds, for this treatment causes no increase in amino nitrogen values from tissues incubated anaerobically. In fact, after autoclaving, the solutions are often cloudy, probably from a slight further precipitation of protein. The amount, however, is negligible and is included in the non-protein nitrogen values. By the Van Slyke procedure, under our conditions, 40 per cent of the nitrogen present as ammonia is estimated. 'It was therefore necessary to subtract 40 per cent of the ammonia nitrogen to obtain the true amino nitrogen values.[1] The protein nitrogen listed in Table I was obtained by subtracting the non-protein from the total nitrogen, and represents the amount precipitated by the trichloroacetic acid at room temperature.   30 per cent of this protein nitrogen is due to red cells and a few liver cells which separate from the slices.   70 per cent is due to protein in solution.

Fig. 1 shows the change in all these values with time for kidney and liver slices, both anaerobically and aerobically. To obtain the initial values, the contents of one flask were removed without incubation and analyzed. Certain effects, which can also be seen in Table I, are immediately obvious, and they can be summarized as follows. The anaerobic condition increases the protein loss from the cells of both kidney and liver; it increases slightly the non-protein nitrogen in kidney and decreases it slightly in liver; it markedly increases the amino nitrogen in kidney and decreases it in liver; and finally, it decreases the ammonia nitrogen in kidney and increases it in liver. No variation from this pattern was observed in over forty experiments with as many animals. The aerobic

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[1]Hamilton (1) has shown that both liver and kidney contain considerable quantities of glutamine. It is difficult to estimate the exact contribution of glutamine to the ammonia and amino nitrogen values. Glutaminase should be active aerobically and anaerobically and therefore some of the estimated ammonia undoubtedly comes from glutamine hydrolyzed during the course of the experiment. Any unchanged glutamine would be converted to pyrrolidonecarboxylic acid by heating in acid and thus the amide nitrogen would also be estimated as ammonia. Therefore the ammonia nitrogen values listed in Table I include all the amide nitrogen of glutamine except that fraction which, after hydrolysis by glutaminase, has been converted to other compounds. Any glutamic acid formed during the experiment will participate to an unknown extent in transamination. Its contribution to the amino nitrogen values is not predictable. It should be emphasized that the ammonia and amino nitrogen values are the result of a large number of metabolic processes.

TABLE I

Effect of Various Substances on Aerobic and Anaerobic Nitrogen Metabolism of Kidney and Liver

The slices were incubated 3.5 hours at 37°. The volume in each flask was 4.0 cc. The results are expressed in mg.

Compound added	Kidney								Liver							
	Aerobic				Anaerobic				Aerobic				Anaerobic			
	Protein N	Non-protein N	NH2-N	NH3-N	Protein N	Non-protein N	NH2-N	NH3-N	Protein N	Non-protein N	NH2-N	NH3-N	Protein N	Non-protein N	NH2-N	NH3-N
4.0 mg. pyruvate	1.12	0.67	0.07	0.46	2.73	0.69	0.22	0.21	1.47	0.61	0.17	0.10	3.04	0.51	0.12	0.14
	1.12	0.52	0.15	0.25	2.41	0.61	0.25	0.10	1.10	0.65	0.20	0.08	2.33	0.39	0.15	0.12
4.0 mg. glucose	1.02	0.65	0.11	0.45	3.60	0.67	0.23	0.19	1.44	0.66	0.22	0.07	3.55	0.50	0.13	0.13
	0.81	0.66	0.11	0.38	3.41	0.67	0.26	0.17	1.34	0.63	0.19	0.08	3.68	0.47	0.12	0.13
4.0 mg. fumarate	1.04	0.58	0.02	0.45	2.02	0.61	0.22	0.20	1.01	0.58	0.19	0.09	2.46	0.42	0.10	0.13
	1.23	0.49	0.09	0.28	2.17	0.61	0.25	0.19	1.24	0.55	0.17	0.10	2.44	0.44	0.09	0.12
4.0 mg. avertin	0.89	0.51	0.06	0.35	2.11	0.57	0.25	0.11	1.06	0.52	0.21	0.07	2.21	0.40	0.12	0.06
	1.07	0.43	0.07	0.29	2.22	0.56	0.24	0.16	1.35	0.37	0.09	0.06	2.16	0.34	0.10	0.09
0.5 mg. p-phenyline-diamine	1.02	0.50	0.02	0.40					1.02	0.65	0.17	0.06				
	1.41	0.59	0.03	0.32					1.06	0.52	0.12	0.06				
0.4 mg. NaCN	2.75	0.67	0.26	0.19					2.65	0.38	0.07	0.12				
	0.91	0.56			2.37	0.65			1.47	0.59			2.52	0.42		
4.0 mg. l-alanine	0.98	0.46			2.26	0.45			1.40	0.42			2.64	0.40		
4.0 mg. l-methionine	0.92	0.36			2.45	0.38			1.62	0.34			2.68	0.40		
2.0 mg. (NH4)2SO4	0.94	0.30			2.30	0.40			1.50	0.40			2.60	0.30		
Hypertonic NaCl	1.10	0.55	0.05	0.42	2.11	0.61	0.28	0.11	1.19	0.50	0.17	0.08	1.90	0.47	0.11	0.12
"	1.36	0.61	0.07	0.37	2.60	0.60	0.26	0.12	1.15	0.50	0.13	0.05	1.96	0.50	0.11	0.09
Hypotonic "	0.97	0.55	0.08	0.39	2.00	0.61	0.28	0.11	0.95	0.48	0.18	0.07	1.91	0.46	0.10	0.11
"	1.09	0.64	0.02	0.47	2.11	0.70	0.24	0.24	1.88	0.65	0.20	0.12	3.25	0.50	0.14	0.12
Fasted 4 days	0.95	0.63	0.06	0.39	2.05	0.67	0.21	0.19	1.73	0.73	0.20	0.14	3.47	0.55	0.14	0.17

protein loss is probably in part an artifact due to the absence of protein in the solution and the unavoidable trauma from shaking the vessels.
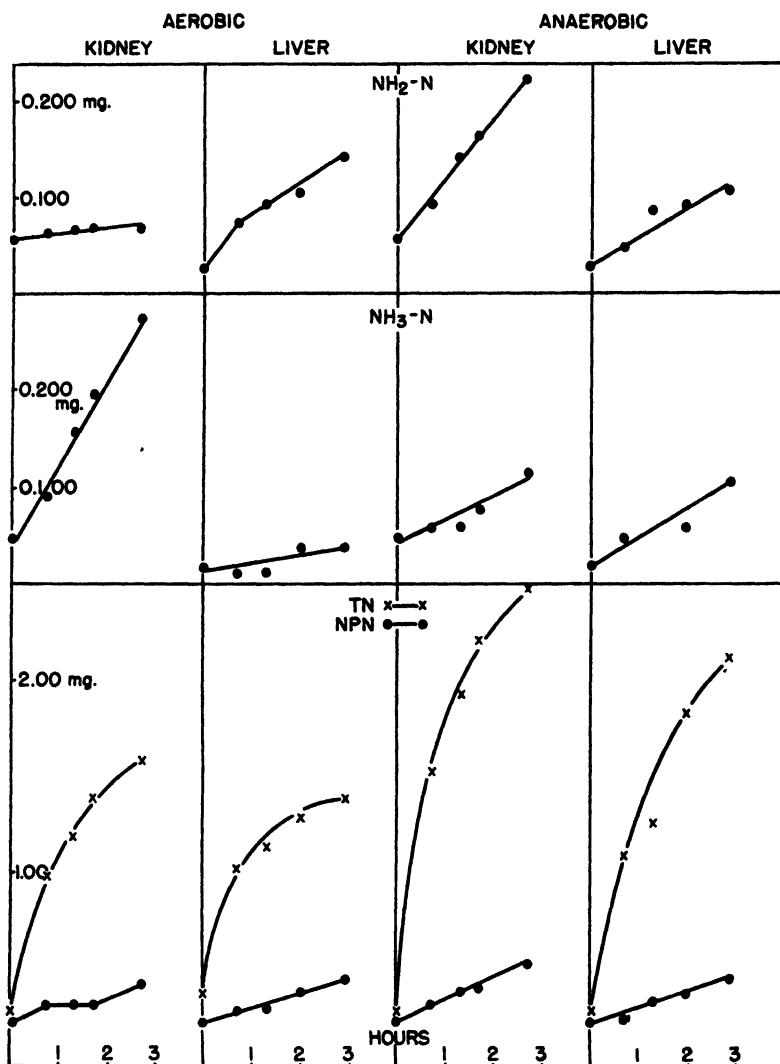
FIG. 1. The changes with time in the various nitrogen fractions of 300 mg. of liver and kidney slices under aerobic and anaerobic conditions.

It is very little affected by hypertonic and hypotonic solutions (1.8 and 0.45 per cent NaCl with all the other constituents present in normal concentrations); nor is it lowered appreciably by 4 days fasting of the

animal before removal of the tissues, when, presumably, some of the
so called labile protein of the liver has disappeared. Absence of calcium
from the solution increases the aerobic protein loss, indicating that the
permeability of the cell membrane is, in part, a limiting factor; and an
increase in this permeability under anaerobic conditions probably accounts
for the greater protein loss.

In kidney the non-protein nitrogen is slightly higher anaerobically, but
the difference is so small that it would not be significant if it were not a
consistent finding. On the other hand, the amino nitrogen is usually more
than doubled anaerobically, while the ammonia nitrogen is markedly
decreased. There is thus a reciprocal relation between these two and it is
apparent that the precursor of most of the aerobic ammonia is a substance
which requires oxidation before the ammonia is liberated. There is some
anaerobic ammonia production but it is relatively small and may not be
significant.

In the liver the situation is exactly the reverse. The non-protein and
amino nitrogen are always less under anaerobic conditions. There is
thus not only no hydrolysis of protein but actually an inhibition of processes
which are involved in the formation of non-protein nitrogen material.
The anaerobic ammonia nitrogen is always higher than the aerobic, for
under these conditions urea production is stopped and any ammonia formed
tends to remain as such. The source of the anaerobic ammonia is unknown
but may be glutamine.

Table I summarizes the effect of a number of substances on the various
nitrogen fractions. Pyruvate has two marked effects. It decreases the
protein loss from the liver anaerobically and to a less extent also in the
kidney. This decrease occurs whether the protein loss is high or low. (In
the control experiments listed in Table I, it is the protein loss that shows
the greatest variations. All the other figures are remarkably constant.)
Pyruvate decreases both the aerobic and anaerobic ammonia nitrogen in
kidney, causing a corresponding increase in the amino nitrogen, but also
some decrease in non-protein nitrogen. Glucose, on the other hand, is
without any significant effect. Pyruvate in its effect on anaerobic protein
loss may be acting as a hydrogen acceptor. If that is so, its action is
nevertheless highly specific, for fumarate has no effect on protein loss.
The latter will, however, reduce the aerobic ammonia production in kidney
and cause some increase in amino nitrogen and decrease in non-protein
nitrogen. Amino acids and ammonia very definitely decrease the non-
protein nitrogen. This result is obtained by subtracting the nitrogen added
as amino acid or ammonium sulfate from the total non-protein nitrogen
estimated. The reduction is significant in all cases except in the anaerobic
liver. It is therefore probable that free ammonia as well as amino acids

prevents the normal breakdown of protein. The corrections in the amino and ammonia nitrogen values when nitrogen compounds are added are so large that the results are not significant.

Avertin belongs to the class of halogenated organic compounds that may cause liver damage. Its main action is on the liver aerobic non-protein nitrogen and amino nitrogen, both of which are decreased. It causes a slight increase in aerobic protein loss in liver and kidney and a small depression of aerobic ammonia production in kidney. Its preferential action therefore is on the liver, where it suppresses the production of non-protein nitrogen and in particular the formation of amino groups. Cyanide has exactly the same effect as anaerobiosis, and when it is added to slices incubated anaerobically it has no added effect. Therefore, it does not increase catheptic activity in kidney or liver under these conditions. *p*-Phenylenediamine is also a respiratory inhibitor and its effects are similar to those of cyanide, but are less marked.

All the above experiments were done at pH 7.4 where autolysis occurs more slowly than in acid solutions. In order to test the extent of autolytic activity at pH 7.4 and under identical conditions liver and kidney cell suspensions were made by grinding in a mortar with sand. The initial amino nitrogen in the kidney suspension was 0.258 mg. and after 3 hours aerobic incubation rose to 0.528 mg. The corresponding values for liver were 0.226 mg. and 0.406 mg. Cyanide and anaerobiosis did not increase these figures and this indicates that they are the result of proteolytic activity not dependent on reducing agents such as sulfhydryl groups. The existence of this type of autolysis was shown by Bailey *et al.* (2) for hog liver. The protein hydrolyzed at pH 7.3 is, according to Luck *et al.* (3), a globulin II. Thus broken cell suspensions show definite autolysis under conditions in which tissue slices show little evidence of it.

Experiments with heart slices show that this tissue is much less active than liver or kidney in the production of ammonia, amino, and non-protein nitrogen. More protein is lost from the slices, possibly because of the syncytial structure. Anaerobic conditions, hypotonicity, absence of calcium, and avertin have minimal effects. If the cells are completely destroyed autolysis occurs.

<center>DISCUSSION</center>

Under the conditions of the experiments it is not possible to say how much the aerobic protein loss from the cells is due to trauma and how much is due to normal diffusion. It is, however, evident that anaerobic conditions greatly increase protein loss and that this is the only anaerobic effect common to both liver and kidney. It seems probable that a similar protein loss from cells to intracellular fluid occurs *in vivo* when the blood

supply to an area is occluded. This loss apparently is the first reaction, for it occurs *in vitro* when there is no evidence of any autolysis. When autolysis occurs *in vivo*, it probably does so only after a considerable portion of the protein has leaked out of the cell. In the above experiments about one-third of the dry weight of the tissue slice is lost as protein to the surrounding fluid under anaerobic conditions lasting 3 hours.

SUMMARY

1. The protein, non-protein, amino, and ammonia nitrogen values have been estimated after incubating rat liver and kidney slices under various conditions.

2. Anaerobic conditions in the kidney cause an increased protein loss, a slight increase in non-protein nitrogen, a large increase in amino nitrogen, with a corresponding decrease in ammonia nitrogen.

3. Anaerobic conditions in the liver cause an increased protein loss, a decrease in non-protein nitrogen, a decrease in amino nitrogen, and an increase in ammonia nitrogen, which results, except for the protein loss, are exactly opposite from those in the kidney.

. 4. The effect of pyruvate, glucose, fumarate, amino acids, and various drugs on these patterns is described.

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AN ELECTROPHORETIC ANALYSIS OF CHANGES PRODUCED IN BLOOD SERUM AND PLASMA PROTEINS BY HEAT IN THE PRESENCE OF SUGARS*

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Van der Scheer and associates (1), using the electrophoretic procedure, demonstrated the formation of a colloidal component C in normal horse serum that had previously been heated to 65°. ·As Beilinsson (2) and others (3) had shown that the coagulation of egg albumin by heat could be prevented by treating the albumin solution with sugars and sugar alcohols, it was thought that a similar treatment of blood serum and plasma might also prevent coagulation and even prevent the formation of the C component. In a preliminary experiment (4) in which bovine plasma was saturated with d-glucose, heat coagulation and the formation of the C component were prevented.

The present study was undertaken for the purpose of comparing the effects of d-galactose, d-fructose, d-mannose, l-arabinose, d-xylose, sucrose, and d-mannitol as well as d-glucose in plasma and serum on the formation of the C component. The change or absence of change in bovine blood serum or plasma was followed electrophoretically by the moving boundary method of Tiselius (5).

EXPERIMENTAL

The plasma and serum samples studied were either saturated or half saturated with the sugars or mannitol at room temperature. They were placed in Erlenmeyer flasks, stoppered, and placed in a constant temperature water bath. Heating was then carried out under the conditions noted in Table I. The amount of precipitate formed during the period of heating varied from no visible precipitate when certain of the sugars were employed to nearly 100 per cent precipitation of the plasma and serum proteins in the case of the controls. Any precipitate formed during the period of heating was removed by centrifugation. The sugar or mannitol

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* Journal article No. 777 (new series) of the Michigan Agricultural Experiment Station.

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was removed by dialysis against distilled water. The protein concentration of the sample was determined and adjusted to that used for study before electrophoresis experiments were carried out.

TABLE I

Heating Experiments

Experiment No.	Fig. No.	Sample	Material added	Time exposed and temperature
1	1	Bovine plasma	None	Control
2	2	" "	Saturated, d-glucose	"
3	3	" "	None	1 hr. at 65°
4	4	" "	Half saturated, d-glucose	1 " " 65°
5	5	" "	Saturated, d-glucose	1 " " 65°
6		" "	None	1 " " 56°
7		" "	Half saturated, d-glucose	1 " " 56°
8		" "	Saturated, d-glucose	1 " " 56°
9		" serum	None	Control
10		" "	"	1 hr. at 65°
11		" "	Saturated, d-galactose	1 " " 65°
12		" "	" l-arabinose	1 " " 65°
13		" "	" sucrose	1 " " 65°
14		" "	" d-fructose	1 " " 65°
15	6	" "	" lactose	1 " " 65°
16		" "	" d-mannitol	1 " " 65°
17		" "	" d-xylose	1 " " 65°
18	7	Fractionated plasma	None	Control
19	8	" "	"	1 hr. at 65°
20		" "	Half saturated, d-glucose	1 " " 65°
21		" "	Saturated, d-glucose	1 " " 65°
22	9	" "	None	Control
23	10	" "	"	1 hr. at 65°
24		" "	Half saturated, d-glucose	1 " " 65°
25	11	Bovine plasma	None	24 hrs. " 56°
26	12	" "	"	48 " " 56°
27	13	" "	"	5 days " 56°
28	14	" "	Saturated, d-glucose	7 " " 56°
29		" "	None (supernatant from Experiment 30)	
30	15	C component	None	

Electrophoretic examinations were carried out in a manner described previously (6). In all cases the samples to be analyzed were dialyzed against large volumes of diethylbarbituric acid-sodium diethyl barbiturate buffer of pH 8.6 and ionic strength 0.1. Electrophoresis of the dialyzed samples was carried out under the conditions shown in Table II.

For purposes of calculation, the areas of the albumin, α_1- and α_2-globulins, and the C component when present were determined in arbitrary planimeter units. Since the C component had about the same mobility as

TABLE II
Electrophoresis Experiments

Experiment No.	Fig. No.	Electro-phoresis time	Potential gradient	Protein concentration	Mobility of C component $\times 10^{-5}$	Relative concentrations	
						Globulin $(\alpha_1 + \alpha_2)$ / Albumin	C component / Albumin
		sec.	*volts per cm.*	*per cent*	*sq.cm. per sec per volt*		
1	1	10,100	6.63	2		0.394	
2	2	10,500	6.38	2		0.380	
3	3	10,000	6.56	2	5.08		3.70
4	4	10,000	6.45	2	4.76		0.710
5	5	10,000	6.90	2		0.390	
6		10,300	6.53	2	5.10		0.750
7		10,000	6.38	2		0.500	
8		12,000	5.46	2		0.469	
9		10,000	5.50	2		0.398	
10		10,000	6.88	2	4.50		8.97
11		10,000	7.35	2	4.79	0.257	
12		10,000	7.13	2			
13		10,000	6.99	2		0.325	
14		10,000	7.16	2		0.303	
15	6	10,100	6.95	2	5.25		3.66
16		10,000	7.30	2	4.94		4.06
17		11,000	7.18	2			
18	7	10,000	6.52	0.66		0.393	
19	8	10,200	6.96	1	5.63		2.30
20		11,000	6.95	1	5.32		0.752
21		10,200	6.73	1		0.443	
22	9	6,000	6.32	1		0.182	
23	10	11,000	6.31	1	6.77		1.66
24		10,000	6.21	1			
25	11	10,000	6.65	2	4.15		1.18
26	12	10,000	6.51	2	4.79		1.90
27	13	10,000	6.30	1.3	4.64		0.95
28	14	10,200	6.63	1.37	5.10		0.79
29		10,200	6.38	2	5.11		
30	15	5,000	6.43	0.33	4.68		3.66

the α-globulins, it often masked them when present. Therefore, it was decided to determine the α_1- plus α_2-globulin-albumin ratio where discernible and the C component-albumin ratio in the other cases. A normal value for the former ratio would indicate the absence of C component and a value higher than normal, its presence.

An electrophoretic pattern of normal bovine plasma is shown in Fig. 1. A pattern of the same plasma is shown in Fig. 2 after having been saturated with d-glucose and the sugar removed by dialysis. The pattern resembles that of normal plasma and the α-globulin-albumin ratios of 0.394 and 0.380 were in good agreement.

The pattern containing the C component shown in Fig. 3 was obtained after heating a sample of the same plasma at 65° for 1 hour. A C component-albumin ratio of 3.70 was observed.
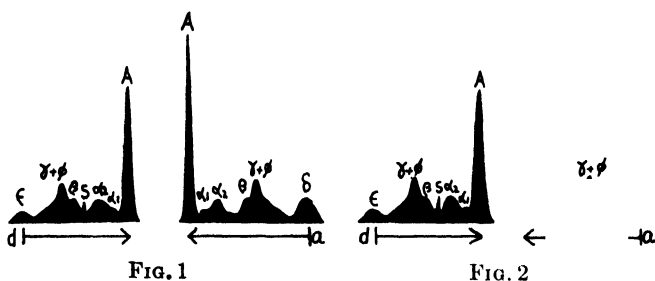
FIG. 1. Electrophoresis pattern of normal bovine plasma.

FIG. 2. Bovine plasma saturated with d-glucose and the sugar removed by dialysis
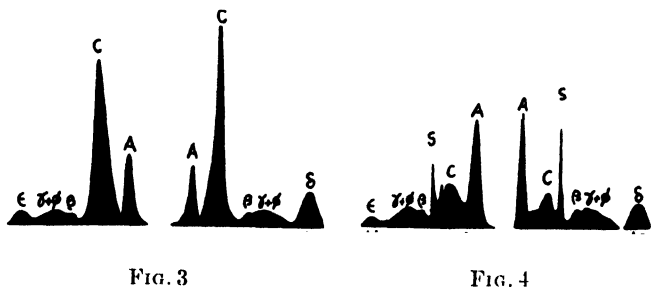
FIG. 3. Bovine plasma heated at 65° for 1 hour.

FIG. 4. Bovine plasma half saturated with d-glucose and heated at 65° for 1 hour.

Fig. 4 shows the effect of heating the plasma under the same conditions when half saturated with d-glucose. The C component-albumin ratio decreased to 0.71. It is of interest to note that the β-boundary disturbance in this case was observed as two spikes in the descending pattern and as one in the ascending. It is possible that heating alters the lipid-protein complex which is considered the cause of the β-boundary disturbance (7).

An electrophoretic pattern of normal bovine plasma after having been saturated with d-glucose and heated is illustrated by Fig. 5. The ascending and descending patterns show little change from the normal. The boundary disturbance appears on both patterns. The fibrinogen-albumin ratio was less than that recorded in normal plasma.

In Table II, Experiment 6, are shown the results obtained when bovine plasma was heated at 56° for 1 hour. The C component-albumin ratio in this case was 0.750 as compared with the value of 3.70 observed under similar circumstances at 65° in Experiment 3.   When bovine plasma was half saturated or saturated with d-glucose and heated at 56° for 1 hour, the formation of the C component was suppressed.   The α-globulin-albumin ratio was 0.500 and 0.469 (Experiments 7 and 8, Table II).

Further experiments were carried out at 65° with normal bovine serum and serum saturated with other sugars and with d-mannitol.   Normal bovine serum (Experiment 9) gave an α-globulin-albumin ratio of 0.398. The C component was formed when a sample of the serum was heated at 65° for 1 hour.   The C component-albumin ratio was 8.97.   When serum
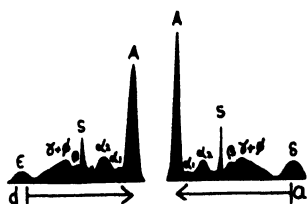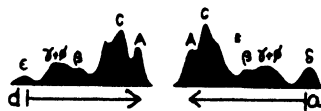
FIG. 5.   FIG. 6.

FIG. 5. Bovine plasma saturated with d-glucose and heated at 65° for 1 hour.
FIG. 6. Bovine serum saturated with lactose and heated at 65° for 1 hour.

was saturated with d-galactose and heated at 65° for 1 hour, the electrophoretic patterns do not show α_1-globulin or the C component.   The α-globulin-albumin ratio was 0.257 (Experiment 11).

When serum was heated in the presence of l-arabinose, the formation of the C component was not completely suppressed.   The C component failed to separate from the albumin sufficiently to calculate the ratio (Experiment 12).

Sucrose and d-fructose completely inhibited the formation of C component.   When lactose (Fig. 6), d-mannitol, and d-xylose were employed (Experiments 15, 16, and 17), the C component appeared as two overlapping components.   In the case of d-xylose, the C component failed to separate from the albumin sufficiently to calculate the ratio.   Qualitatively, the changes in the electrophoretic patterns in Experiment 17 in which d-xylose was employed appeared to be of the same magnitude as was observed in Experiment 12 with l-arabinose.

Critical examination of the heated plasma samples shown in Figs. 3, 4, and 5 shows a decrease in the concentrations of β- and γ-globulin and fibrinogen.   It might be surmised that the C component forms at the expense of these three components.   However, Experiments 9 through 17 carried out

with bovine serum show that the C component can form in the absence of fibrinogen.

In an attempt to obtain more information on the origin of the C component, normal bovine plasma was fractionated with cold 40 per cent ethanol according to the method described by Cohn *et al.* (8). The procedure employed consisted of placing normal bovine plasma to be fractionated in an agitated cellophane membrane which could be suspended in an ethanol solution. The ethanol was then added to the plasma by diffusion. In order to prevent denaturation of the plasma proteins by the ethanol, the entire operation was carried out at 0° and the gradient of ethanol within and without the membrane was maintained as small as convenient. In practice the samples were equilibrated against 10, 20, and 30 per cent ethanol and finally against 40 per cent ethanol. No attempt was made to adjust the pH of the ethanol solution or the plasma. The fraction remaining in solution after equilibrium against cold 40 per cent ethanol was dia-

FIG. 7.   FIG. 8.
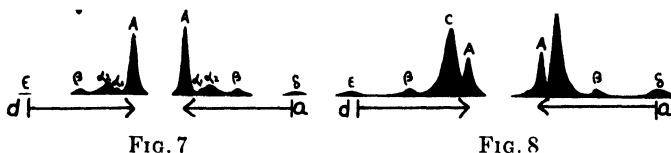
FIG. 7. Bovine plasma fraction soluble in 40 per cent ethanol at 0°.

FIG. 8. Bovine plasma fraction soluble in 40 per cent ethanol at 0° after heating for 1 hour at 65°.

lyzed against several changes of potassium phosphate buffer of pH 7.2 and ionic strength 0.1. The electrophoretic pattern of this fraction is shown in Fig. 7. It appeared to be a mixture of albumin and α- and β-globulins. The dialyzed fraction was divided into three portions. One was heated at 65° for 1 hour, one was half saturated, and one was saturated with d-glucose and heated as the untreated portion. Fig. 8 shows that the C component appears in the absence of either fibrinogen or γ-globulin. Its appearance was partially suppressed in the other two treated portions of the fractionated plasma.

A more complete fractionation of normal bovine plasma, in the manner described above, with 52 per cent ethanol at −5°, resulted in the soluble fraction shown in Fig. 9. This fraction was a mixture of albumin and α-globulins. It was dialyzed as described previously and divided into three portions. One was heated at 65° for 1 hour, one half saturated, and one saturated with d-glucose and heated as the untreated portion. As shown in Fig. 10, the C component formed but failed to separate completely from the albumin component. Its electrophoretic mobility was

6.77×10^{-5} sq. cm. The α_2-globulin peak is absent. The electrophoretic patterns obtained on the two d-glucose-treated portions did not show the C component.

Further attempts to determine the origin of the C component were carried out by heating samples of bovine plasma at 56° for extended periods of time. Fig. 11 shows the electrophoretic patterns obtained after heating bovine plasma for 24 hours. The C component-albumin ratio was 1.18. The albumin, fibrinogen, and globulin concentrations decreased. In a similar experiment at 56° heating for 48 hours caused the changes shown in
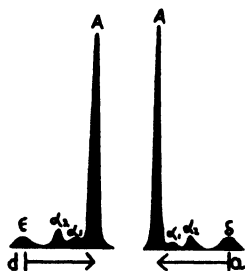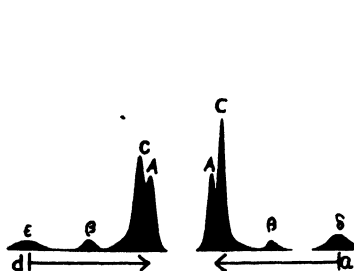
FIG. 9   FIG. 10

FIG. 9. Bovine plasma fraction soluble in 52 per cent ethanol at −5°.

FIG. 10. Bovine plasma fraction soluble in 52 per cent ethanol at −5° after heating for 1 hour at 65°.
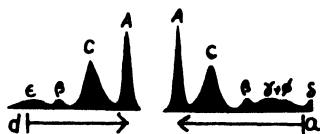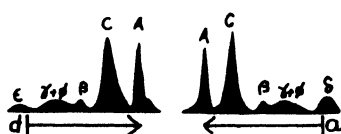
FIG. 11   FIG. 12

FIG. 11. Normal bovine plasma heated at 56° for 24 hours.

FIG. 12. Normal bovine plasma heated at 56° for 48 hours.

Fig. 12. The C component-albumin ratio was 1.90. Fig. 13 was obtained after heating plasma for 5 days at 56°. At the end of this period of heating the sample contained a large amount of precipitate and was very viscous. It was found that under these conditions 80 per cent of the plasma protein had been rendered insoluble. Centrifugation was used to clarify the sample. Most of the C component was removed through this treatment. A C component-albumin ratio of 0.95 is obviously not a true indication of the amount of C component formed. In this case, there was a further decrease in albumin, fibrinogen, and globulin concentrations. The presence of a new component migrating ahead of the albumin fraction is also shown in Fig. 13.

The formation of the C component was diminished in plasma that had been saturated with d-glucose and heated for 7 days at 56°. The plasma remained clear, though it may be observed from the electrophoretic patterns (Fig. 14) that physicochemical changes occurred during the period of heating. The C component-albumin ratio was 0.79.

Van der Scheer and associates (1) were able to separate the C component completely by dialyzing heated serum in distilled water and adjusting to pH 6.0. The C component was then removed by centrifugation and was dispersed in alkaline saline solution. An electrophoretic examination of
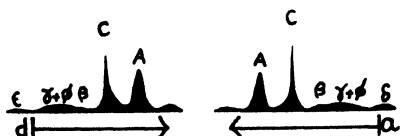
FIG. 13. Normal bovine plasma heated at 56° for 5 days
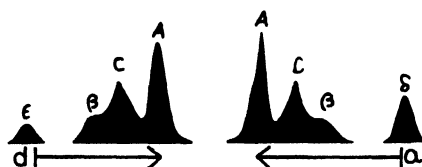
FIG. 14. Bovine plasma saturated with d-glucose and heated at 56° for 7 days
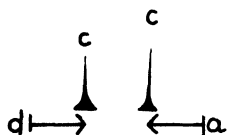
FIG. 15. C component isolated from heated bovine plasma

this dispersion showed the C component to be homogeneous. Their procedure was applied to heated bovine plasma by the authors. An electrophoretic examination of the dispersion produced the patterns shown in Fig. 15. The C component appeared homogeneous and had a mobility of the order observed previously in the heated samples. Only a small portion of the C component was removed by following the method employed by van der Scheer and coworkers.

DISCUSSION

Van der Scheer and associates (1) found that heating serum albumin by itself did not result in C component formation under conditions which caused it to appear in a serum globulin fraction that was free of albumin. These authors explained its formation by assuming that the globulin in

serum became denatured during heat treatment and aggregated to form a colloidal suspension of particles fairly uniform with respect to the electrical charge they carried. The fact that serum albumin by itself did not produce the C component when heated under conditions which caused its formation in serum, with a corresponding decrease in the albumin concentration, was explained by assuming that albumin was bound up in the colloidal C component.

The present study shows that the C component is formed when bovine serum or plasma and certain fractionated bovine plasma samples are heated. As yet a globulin-free albumin fraction has not been studied. However, preliminary experiments with bovine plasma and serum globulin fractions have shown the presence of a new component following heating. The present studies on various fractionated bovine plasma samples show that the C component forms in the absence of fibrinogen and γ- and β-globulin.

The fact that there was such a wide variation in the electrophoretic mobility of the C component (Table II) in spite of its apparent electrophoretic homogeneity may be due to different comcentrations of albumin in the colloidal, denatured protein-albumin complex.

No satisfactory explanation of the mechanism of the inhibiting action of the various substances studied against the formation of the C component is possible at present. Earlier work (3) indicated that the inhibiting action could not be explained on the formation of "symplexes," an idea suggested by von Przylecki and Cichocka (9). Schubert (10) prepared crystalline compounds of several sugars with cysteine, but was unable to prepare a crystalline derivative of cysteine with d-fructose, so concluded that an aldehyde group was involved. However, in the present work both sucrose and d-mannitol have shown some prevention of the formation of the C component, and earlier work (3) has shown that these same substances and also d-fructose have inhibited the formation of sulfhydryl groups during the heating of egg albumin. Ballou, Boyer, Luck, and Lum (11) believe the action of various anions, such as the caprylate ion, which are active at even higher temperatures than those used in the present work, is due to an association by electronic attraction of the (COO^-) group with the exposed amino groups $(—NH_3^+)$, the latter formed as a result of the unfolding of the albumin molecule. This attraction, they point out, along with the influence of the non-polar portion of the anion with the non-polar part of the amino acid side chains, would keep the protein in solution. There is no evidence to show that sugars would act in a similar manner. From a chemical viewpoint it is not clear why certain of the substances used in the present work failed to prevent the formation of the C component, whereas others gave essentially 100 per cent inhibition.

SUMMARY

d-Glucose inhibited the formation of the C component when bovine plasma was heated under conditions that caused its formation in the absence of *d*-glucose.

d-Galactose, *l*-arabinose, sucrose, *d*-fructose, lactose, *d*-mannitol, and *d*-xylose all showed some degree of inhibiting action against the C component formation in heated bovine serum.

Aging in the presence of *d*-glucose did not alter the electrophoretic patterns of bovine plasma.

Studies on bovine plasma fractions gave further evidence of the inhibiting action of *d*-glucose against C component formation but did not explain the mechanism of its formation. The C component was formed by heating an albumin-*α*-globulin fraction of bovine plasma.

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STUDIES ON THE ENZYMATIC SYNTHESIS OF DEXTRAN FROM SUCROSE*

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The syntheses of dextrans (1–5) and levans (6–10) from sucrose by enzymes from certain bacteria and the syntheses of glycogens and starches from glucose-1-phosphate by phosphorylases from yeast (11) and animal (12–15) and plant (16, 17) tissues have a fundamental point of similarity in that, with all of them, the substrate contains the basic unit of the final polymer product in the form of a glycoside radical (5). A point of difference from the glycogen and starch syntheses is that phosphorylated compounds apparently are not required in the syntheses of dextrans and levans (5, 8). A further distinction is that the enzymatically synthesized dextrans and levans that we have studied (1, 2, 4, 9) are serologically active; this feature, although not representing a fundamental difference in the syntheses themselves, is of special interest to the bacteriologist, and has been utilized to particular advantage in examining the question of the likeness between the polysaccharides synthesized by the cell-free enzymes and the natural polysaccharides formed in the living bacterial cultures (2, 4).

The present paper deals with some aspects of the kinetics of dextran formation from sucrose by enzymes from a strain of *Leuconostoc mesenteroides*. Previous papers (1, 2, 4, 5) included data indicating that this enzymatic reaction consists of the conversion of n molecules of sucrose to a polymer of n glucose anhydride units plus n molecules of fructose, and also data showing that the enzymatically synthesized dextran agreed in serological as well as in chemical properties with the dextran elaborated in living cultures of the bacteria from which the enzymes were derived. The present object is to present data on the equilibrium point, velocity constants, and other aspects of the reaction, which could be studied more adequately with the more potent enzyme preparations described in this paper than with the enzyme preparations utilized in the previous studies.

EXPERIMENTAL

Preparation of Enzymes—5 liters of a culture of *Leuconostoc mesenteroides* (the previously utilized B strain) represented the usual starting material. The culture medium comprised sucrose 4 per cent, Bacto-peptone 1.5

per cent, NaCl 0.5 per cent, and Na_2HPO_4 0.1 per cent; it was heavily seeded and was incubated for 2 days at 23°. The final cultures were opalescent and viscous, and had a pH of from 4.5 to 4.8. To each liter of culture fluid, decanted from the gum that was deposited at the bottom of the flasks, 370 gm. of ammonium sulfate were added. The mixture was centrifuged for 1 hour at 1500 R.P.M., and the supernatant fluids which contained most of the preformed dextran were decanted from the precipitates which contained the enzyme. The precipitates were drained in the cold for 30 minutes, and were further freed from preformed polysaccharide material by washing three times with 250 ml. of half saturated ammonium sulfate in 0.1 per cent acetic acid. For each washing, the sticky precipitate was carefully worked from the bottoms and sides of the centrifuge tubes into the acid wash fluid with the aid of a rubber policeman; the suspension was then centrifuged and the wash fluid decanted. The final washed precipitate, which contained large numbers of bacterial cells in addition to the enzyme, was drained and then extracted with 100 ml. of 0.025 M citrate buffer (pH 6.3). Following a preliminary centrifugation, the extract, readjusted if necessary to pH 6.3 with ammonia, was centrifuged for 2 hours at 2500 R.P.M. in collodion tubes set in a 14 inch angle head. The resulting solution, which was faintly opalescent but entirely free of bacteria, was treated with an equal volume of 1.0 M acetate buffer (pH 4.3), stored overnight at 4°, and then centrifuged at high speed in the cold. The precipitate, which contained most of the active enzyme together with considerable material (probably primary proteose) derived from the "peptone" of the culture medium, was taken up in 100 ml. of 0.025 M citrate buffer (pH 6.3), cleared by centrifugation, and stored in a solid CO_2 freezing chamber at approximately −70°.

Enzyme-Substrate Mixtures—The sucrose used as substrate in the experiments was a sample of beet sugar known to be free of dextran; it was used in place of reagent sucrose, because the latter usually contains appreciable amounts of material that has the serological properties of *Leuconostoc* dextran (18, 19). Unless otherwise noted, solutions of the sucrose were prepared in 0.1 M acetate buffer (pH 5.6) immediately before use. The incubation of all enzyme-substrate mixtures was at 23°; after the proper interval the reaction was stopped, usually by diluting the mixtures 5-fold in 0.02 N sodium hydroxide. Immediately thereafter analyses of the enzyme-substrate mixture were made for one or more of the components of the reaction (free reducing sugar, dextran, sucrose).

Analytical Procedures

Reducing sugar was determined "as glucose" in triplicate analyses by the method of Hagedorn and Jensen (20). This procedure was considered

applicable to the determination of the free reducing sugar, which is fructose, as well as to the determinations of dextran and sucrose, because fructose and glucose have essentially the same reducing capacity when assayed by this method.

For the estimation of dextran the sample to be analyzed was diluted 5-fold in 10 per cent sodium acetate and then treated with 1.5 volumes of 95 per cent alcohol. The precipitate, collected by centrifugation of the mixture after it had been in the ice box for several days, was taken up in 1 N sulfuric acid and hydrolyzed in a sealed tube immersed in boiling water for 6 hours. The reducing sugar content of the neutralized hydrolysate was taken to represent the dextran content. That basis was used, even though a polyglucoside theoretically would be calculated as 90 per cent of the amount of reducing sugar liberated on hydrolysis, because reducing sugar values obtained (2, 21) following hydrolysis of purified dextrans have been found to be consistently lower than those theoretically expected and to approach more closely the weight of the dextran taken for analysis.

Sucrose was determined in duplicate by a modification of the procedure of Shapiro (22), which involves the destruction of reducing sugars by means of alkali prior to the determination of reducing power of the original and of the inverted solution. For the alkali pretreatment, 1.0 ml. of the enzyme-substrate mixture to be analyzed was heated at 100° for 25 minutes with 1.5 ml. of 0.5 N sodium hydroxide; the alkaline mixture was cooled, neutralized with 0.1 N hydrochloric acid, and brought to 20.0 ml. Next, the reducing sugar content was determined both before and after inversion. Reducing sugars after inversion were measured upon 3.0 ml. portions to which had been added 1.0 ml. of highly potent invertase in 0.1 M acetate buffer (pH 4.6) and, after incubation for 1 hour at 23°, 10.0 ml. of 0.015 N sodium hydroxide. Reducing sugars before inversion were determined in the same way except that the invertase was not added until immediately before the analysis. The sucrose content was calculated as 0.95 of the difference between the reducing sugars before and after inversion.

General Course of Reaction—The over-all action of *Leuconostoc* enzyme upon sucrose can be illustrated by the data from an experiment in which a mixture of equal volumes of enzyme and of sucrose solution was analyzed after various periods of incubation for sucrose, dextran, and free reducing sugar, and also for serological reactivity.

It is evident (Fig. 1) that the conversion of sucrose to dextran and free reducing sugar began without a noticeable lag period, proceeded at a rate that diminished in an orderly way with time, and finally approached completion. Throughout the reaction there was a regular relationship between the dextran and the free reducing sugar produced: on a weight basis, the amount of free reducing sugar always slightly exceeded the

amount of dextran; furthermore, the formation of dextran and reducing sugar was associated at all periods with a corresponding loss of sucrose. These relationships are consistent with the equation

$$nC_{12}H_{22}O_{11} \quad (C_6H_{10}O_5)_n + nC_6H_{12}O_6 \tag{1}$$
$$\text{Sucrose} \qquad \text{Dextran} \qquad \text{Fructose}$$
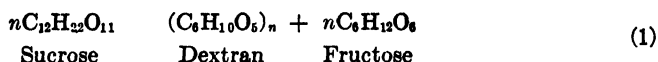
which was suggested earlier (1, 2) as an approximate expression of the reaction of dextran formation by the enzyme from this strain of bacteria.
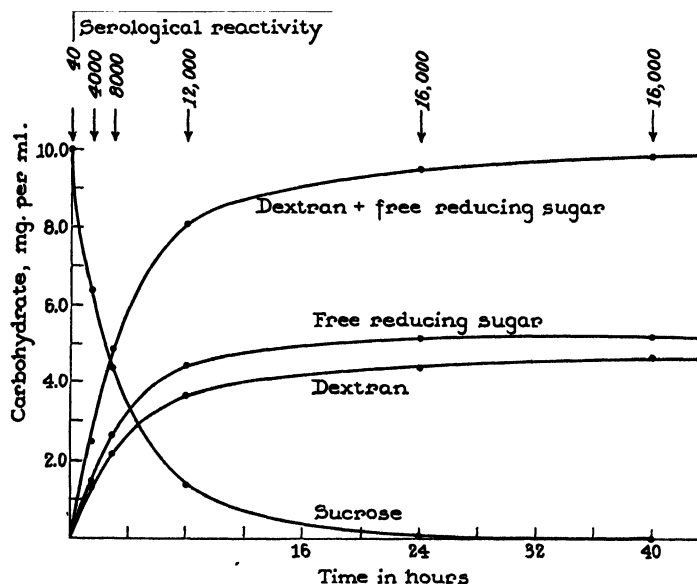

FIG. 1. Time curves for the conversion of sucrose to dextran and free reducing sugar. Serological reactivity represents the highest dilution of the enzyme-sucrose mixture that gave precipitation with a dextran-reactive sample of type 2 pneumococcus antiserum (21, 23).

The possession of serological properties by the enzymatically synthesized dextran is illustrated by the marked increase in serological reactivity which accompanied the conversion of the sucrose to dextran. Detailed data on the specificity of the serological reactions of the enzymatically synthesized dextran have been given in previous papers (2, 4).

Equilibrium—Mixtures of enzyme plus several different concentrations of sucrose were incubated for relatively long periods of time and then analyzed for their contents of sucrose, dextran, and reducing sugar. As shown in Table I, the conversion of sucrose to dextran and free reducing sugar went nearly to completion in all the experiments. That is, the concentrations of sucrose, reducing sugar, and dextran found on analysis

agreed closely with those calculated on the basis that all the sucrose was converted to dextran and fructose[1] according to the previously proposed equation. Although the reaction did not go to absolute completion, the amount of residual sucrose found on analysis was always small and represented only 0.1 to 1.2 per cent of that originally present. (The validity of these low values was proved by the nearly quantitative recoveries of sucrose in control tests in which small amounts (0.5 per cent of that initially present) were added to samples of the mixtures just before their analysis.)

In respect to the minimal amounts of substrate remaining when the reaction approaches equilibrium, the condensation of sucrose to dextran by the *Leuconostoc* enzyme resembles the hydrolysis of sucrose by invertase

TABLE I

Approximately Complete Conversion of Sucrose to Dextran and Fructose by Leuconostoc Enzyme

Sucrose, initial concentration	Time of incubation	Final concentrations of reactants				
		Found on analysis			Expected*	
		Sucrose	Reducing sugar	Dextran	Fructose	Dextran
mg. per ml.	*hrs.*	*mg. per ml.*	*mg. per ml.*	*mg. per ml.*	*mg. per ml*	*mg. per ml.*
5.0	24	0.02	2.60	2.47	2.63	2.37
5.0	48	0.01	2.63	2.42	2.63	2.37
10.0	40	0.01	5.16	4.65	5.26	4.74
50.0	50	0.59	27.0	22.8	26.3	23.7

* Expected on the basis of complete condensation of the sucrose according to Equation 1.

(25), but differs from the enzymatic syntheses of glycogen, starch, and sucrose by phosphorylases, which reactions reach equilibrium long before the substrate is completely utilized (13, 16, 26). Further evidence of the

[1] Direct evidence was previously obtained (2) that fructose is a product of the reaction. The following additional data show that little if any glucose accompanies the fructose. A portion of the enzyme-sucrose mixture from the last experiment in Table I, which was known to be near completion, was treated with 6.3 volumes of alcohol in order to remove the dextran and enzyme material. The supernatant fluid, which contained the reducing sugar, when examined with a Schmidt and Haensch polarimeter made available through the courtesy of Dr. du Vigneaud of the Department of Biochemistry, gave an optical rotation of $\alpha_D^{25} = -0.39°$ ($c = 0.368$ in 80 per cent ethyl alcohol, $l = 2$ dm.), which agreed well with the rotation of crystalline fructose, $\alpha_D^{25} = -0.41°$ ($c = 0.368$ in 80 per cent ethyl alcohol, $l = 2$ dm.). Moreover, the supernatant fluid and a solution of crystalline fructose which showed the same reducing sugar content, when analyzed by the Hagedorn-Jensen (20) method, gave identical fructose values when analyzed by the Roe (24) procedure.

difference between the position of equilibrium in the dextran synthesis and those in the syntheses by phosphorylases is that, with the latter, reversal of the reactions can readily be demonstrated by addition of the enzymes to mixtures of the products, whereas no evidence of reversal was obtained in analogous experiments in which the dextran-synthesizing enzyme was added to mixtures of fructose and dextran. As shown in Table II, no sucrose was formed and no loss in fructose or dextran occurred either in pH 5.6 acetate buffer which represented conditions analogous to those under which the formation of sucrose from glucose-1-phosphate and fructose has been demonstrated (26), or in pH 7.6 phosphate buffer which gave conditions analogous to those under which reversal of glycogen and starch syntheses has been accomplished (13, 16). However, the negative

TABLE II

Attempts to Show Reversal of Reaction of Dextran Synthesis

Buffer utilized	Time of incubation	Analysis of enzyme-substrate mixtures			
		Sucrose	Fructose	Dextran	Serological reactivity*
	hrs.	*mg. per ml.*	*mg. per ml.*	*mg. per ml.*	
0.05 M acetate, pH 5.6	0	<0.01	4.82	4.31	1:20,000
	48	<0.01	4.79	4.32	1:20,000
0.05 " phosphate, pH 7.6	0	<0.01	9.42	8.82	1:40,000
	48	<0.01	9.53	8.82	1:40,000

* Highest dilution of enzyme-substrate mixture that gave precipitation in tests with type 2 pneumococcus antiserum.

results in Table II do not imply that reversal of dextran synthesis might not be demonstrated by some indirect method such as that recently used by Doudoroff and O'Neal (27) in the case of levan synthesis.

Reducing Sugar As Index of Dextran Formation—The data from the experiments of Fig. 1 and Table I, as well as from earlier experiments (1, 2), showed that the amount of reducing sugar formed closely paralleled the extent of conversion of sucrose to dextran. The ratio of reducing sugar to dextran in all experiments averaged 1.14, which agrees well with the ratio of 1.11 expected on the basis of Equation 1. Because of the constancy of this ratio, analysis for reducing sugar was utilized as the measure of dextran formation in the remaining experiments of the present paper, in a way analogous to the use of inorganic phosphate as an index of starch and glycogen formation in systems containing phosphorylase and glucose-1-phosphate. The acceptability of the reducing sugar as a measure of dextran formation was supported by evidence from other experiments

that enzyme preparations from the present strain of *Leuconostoc* cause no appreciable hydrolysis, phosphorolysis, or levan formation from sucrose.

Effect of Substrate Concentration on Initial Rate of Reaction—A series of mixtures was prepared in which 0.2 ml. of enzyme and 0.8 ml. of different concentrations of sucrose in buffer (pH 5.6) were incubated at 23° for 1 hour. The reaction was stopped by the addition of 4.0 ml. of 0.02 N NaOH to the mixtures, after which the content of reducing sugars was
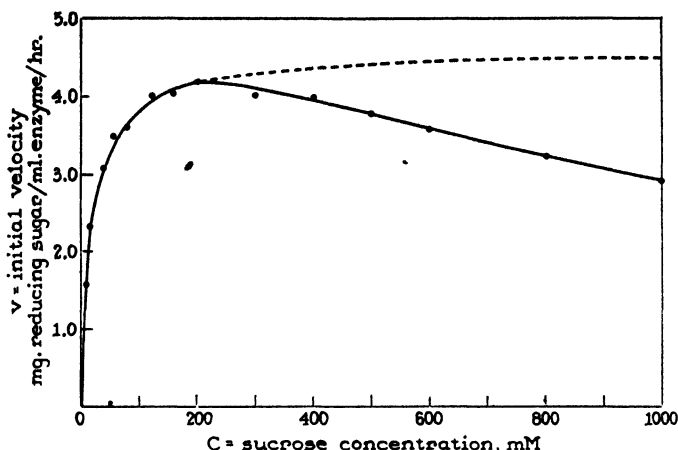

FIG. 2. Effect of substrate concentration on the initial velocity of dextran synthesis. The broken line represents the curve expected from the equation of Michaelis and Menten.

measured. The data are plotted in Fig. 2, along with the theoretical curve expected according to Equation 2 (*cf.* Michaelis and Menten (28)),

$$\frac{V_{max.}\,C}{K_s + C} \tag{2}$$

in which v is the observed velocity of enzyme action (mg. of reducing sugar produced per ml. of enzyme per hour), C is the molar sucrose concentration for velocity v, and $V_{max.}$ is the velocity for full saturation of the enzyme. In constructing the curve based on Equation 2, the value of $V_{max.}$ (4.59 mg. of reducing sugar per ml. of enzyme per hour) was calculated from the data obtained with sucrose concentrations below 200 mM, by application of the graphic method introduced by Lineweaver and Burk (29).

Fig. 2 shows that the velocity of dextran formation rises with increasing substrate concentrations up to about 200 mM, but then declines as the substrate concentration is further increased. This behavior does not completely follow Equation 2 in that velocities lower than the theoretical are found at the higher substrate concentrations. A similar phenomenon

occurs with urease, invertase, and other enzymes. · In the case of invertase, the deviation from the Michaelis and Menten equation has been shown by Nelson and Schubert (30) to be due to the low molecular concentration of one of the reactants (water) in the strong sucrose solutions. The deviation in the case of the *Leuconostoc* enzyme might be due to the same factor; considerable amounts of water unquestionably are "bound" during the formation of the colloidal solution of dextran; so that water perhaps can be regarded as a reactant in the sense that it hydrates the newly synthesized dextran.

TABLE III

Determination of Value of K_s for Dextran-Synthesizing Enzyme

Experiment 1			Experiment 2		
C	v	K_s	C	v	K_s
mM	*mg. per ml.**	*mM*	*mM*	*mg. per ml.**	*mM*
10	1.58	19.1	10	1.58	20.2
20	2.33	19.5	12.5	1.94	18.2
40	3.08	19.7	16	2.10	20.3
60	3 48	19.3	20	2.47	18.6
80	3.60	22.0	25	2.70 ·	19.1
120	4 00	17.7	40	3.30	17.7
160	4.03	22.5	100	4.00	19.0
200	4.20	18.6		4.76 (V_{max}.)	
	4.59 (V_{max}.)				
Average		19.8			19.0

* Mg. of reducing sugar produced per ml. of enzyme per hour.

The concentration of sucrose (K_s) at which the dextran-synthesizing enzyme works at half maximal speed was calculated from the data both of the foregoing and of a second similar experiment. It is evident (Table III) that the K_s was 19 to 20 mM, which is reasonably near the K_s values (16 to 40 mM, 20 to 60 mM) reported for yeast invertase (31) and for levansucrase (6, 10) but definitely higher than that (4.8 to 6.4 mM) reported for glycogen phosphorylase (13, 14).

Determination of Velocity Constants—Two experiments were carried out in which the initial concentrations of sucrose were 1 per cent and 5 per cent respectively; in both, the volume of enzyme was one-half that of the enzyme-sucrose mixture. Graphic analysis of the data indicates that the dextran formation followed a first order course in the experiment with the 1 per cent sucrose, and a zero order course in the experiment with the 5 per

cent sucrose. The first order velocity constant, K_1, and the zero order velocity constant, K_0, were calculated according to Equations 3 and 4

$$K_1 \quad \frac{1}{t} \log_e \frac{S_0}{S_0 - S} \tag{3}$$

$$K_0 \quad \frac{S}{t} \tag{4}$$

where S_0 is the initial sucrose concentration and S is the amount of sucrose converted in time t (*i.e.*, 1.9 times the amount of reducing sugar liberated). Table IV presents the values of K_1 from the data of the experiment with 1

TABLE IV

Calculation of Velocity Constants for Preparation of Dextran-Synthesizing Enzyme

t	Initial sucrose concentration, S_0, 1 per cent			Initial sucrose concentration, S_0, 5 per cent		
	Reducing sugar formed	S	K_1	Reducing sugar formed	S	K_0
hrs.	*mg. per ml.*	*mg. per ml.*	*hrs.*$^{-1}$	*mg. per ml.*	*mg. per ml.*	*mg. per hr.**
0.5	0.45	0.86	0.18	0.78	1.48	5.92
1.0	0.91	1.73	0.19	1.51	2.87	5.74
1.5	1.28	2.43	0.19	2.29	4.35	5.80
2.0	1.64	3.12	0.19	3.06	5.81	5.81
2.5	1.94	3.69	0.18	3.76	7.14	5.71
3.0	2.24	4.26	0.19	4.52	8.49	5.73
3.5	2.50	4.75	0.18	5.04	9.58	5.47
4.0	2.77	5.26	0.19	5.69	10.81	5.41
Average			0.19	●		5.79†

* Mg. of sucrose converted to dextran per ml. of enzyme solution per hour.
† Computed from the data for the first 3 hours only.

per cent sucrose and the values of K_0 from the data of the experiment with 5 per cent sucrose. The K_1 values agreed reasonably well over the entire 4 hour period of the experiment, which represented a time when over 50 per cent of the substrate had been utilized; the K_0 values also agreed well over the first 3 hours which represented a time when 17 per cent of the substrate had been utilized, but as might be expected showed some decline at the later observations. On the basis of the latter velocity constant, the present dextran-forming enzyme can be regarded as 20 times more potent than the preparations used in our earlier studies (1, 2); that is, 1 ml. of the present enzyme converted 5.79 mg. of sucrose to dextran per hour, whereas only 0.3 mg. of sucrose was converted to dextran per hour by 1 ml. of a representative lot of enzyme prepared by the previously described (2) method.

Influence of Added Dextran upon Activity of Enzyme—Data showing that the addition of the preformed polysaccharides has an activating influence upon glycogen- and starch-synthesizing phosphorylases have contributed to the understanding of the mechanism of action of these enzymes (13, 16, 32, 33). Analogous experiments were made to determine whether or not the addition of dextran to enzyme-sucrose mixtures would increase the

TABLE V

Influence of Added Dextran upon Initial Velocity of Dextran Formation

Experiment 1. 1.0 ml. of enzyme, 0.5 ml. of 20 per cent sucrose in 0.2 M acetate (pH 5.6) plus 0.5 ml. of various dilutions of purified dextran; incubated 1 hour at 23°; reaction stopped by addition of 8.0 ml. of 0.02 N NaOH.

Experiment 2. 1.0 ml. of 1:50 enzyme, 0.5 ml. of 20 per cent sucrose in 0.2 M acetate (pH 5.6) plus 0.5 ml. of various dilutions of purified dextran; incubated 3 hours at 23°; reaction stopped by addition of 12.0 ml. of 0.01 N NaOH.

Concentration of added dextran in enzyme-sucrose mixture	Initial reaction velocity*	
	Experiment 1	Experiment 2
mg. per ml.		
0.0	3.76	3.76
0.1	3.68	3.74
0.2	3.64	3.69
0.5	3.58	3.62
1	3.50	3.75
2	3.62	3.70
5	3.64	3.64
10	3.50	3.55

* Mg. of reducing sugar per ml. of enzyme per hour.

activity of the *Leuconostoc* enzyme, although it was realized that the initial presence of small amounts of dextran[2] in the enzyme preparations would

[2] All of the enzyme solutions contain some small amount of material which gives precipitation and complement fixation with antiserums which react with purified dextran. If, as we believe, this material is dextran, the amount present in the enzyme solutions may be roughly estimated to be of the order of 0.01 mg. per ml.; that is, in comparisons made in parallel tests against the same antiserum, the enzyme solutions reacted when diluted 1:40 or 1:80 but not at higher dilutions, whereas the purified dextran from the same strain of bacteria gave positive reactions when diluted as much as 1:4 or 1:8 million. The freeing of the enzyme from these small amounts of serologically reactive material is apparently difficult. We have failed to accomplish it by various treatments with a number of the chemical reagents commonly employed for the purification of the enzymes and proteins, and have failed also in attempts to make the separation by absorption treatment of the enzyme with specific antiserums. These results indicate that small amounts of dextran are closely associated with the enzyme, and suggest the possibility that dextran may actually be an integral part of the enzyme molecule.

complicate the interpretation of the results. The dextran (21) added to
the enzyme-sucrose mixtures was from the same strain of bacteria as that
from which the enzyme came.   •

The data (Table V) show that the addition of purified dextran did not
cause any increase in the initial velocity of dextran synthesis by the enzyme;
there was instead a slight retardation when large amounts were added.
However, since the small amount of the polysaccharide that regularly
accompanies the enzyme[2] might by itself be sufficient to prime the reaction,
failure of the introduction of additional amounts to have an activating
influence does not answer the fundamental question of whether or not
preformed dextran participates in some way in the enzymatic synthesis.

TABLE VI

*Substances without Appreciable Effect upon Synthesis of Dextran from Sucrose by
Leuconostoc Enzyme*

Substance	Final (molar) concentration
Sodium azide	0.025, 0.005, 0 001
" fluoride	0.05, 0.01, 0.002
Potassium cyanide	0.05, 0.01, 0.002
Monoiodoacetic acid	0.025, 0.005
Copper sulfate	0.001, 0.0001, 0.00001
Manganous chloride	0.001, 0.0001
Zinc sulfate	0.001, 0 0001, 0.00001
Silver nitrate	0.0001,* 0.00001, 0.000001
Pyridine	0.01, 0.001
Aniline	0.01, 0.001

* Slight retardation of dextran formation was observed.

Since information on that question is essential for a better understanding
of the mechanism of dextran synthesis, further experiments to explore
this will be performed.

Effect of Various Reagents on Activity of Enzyme—A number of sub-
stances commonly employed in the study of enzyme inhibition were found
to have no effect upon the initial rate of dextran synthesis by the *Leuconostoc*
enzyme in experiments with 5 per cent sucrose at pH 5.6. The substances,
and the concentrations in which they were tested, are listed in Table VI.

The failure of relatively high concentrations of azide, fluoride, cyanide,
or iodoacetate to retard the action of *Leuconostoc* enzyme suggests that the
synthesis of dextran from sucrose is not coupled with or dependent upon
oxidative processes. The lack of appreciable effect of aniline and of Ag^+
and Cu^{++} ions upon dextran formation is in contrast to the pronounced
inhibitory effect of aniline and of Ag^+ ions upon yeast invertase (34) and
of Cu^{++} ions upon glycogen phosphorylase (13).

SUMMARY

1. The activity of a bacterial enzyme which catalyzes the formation of a serologically reactive polysaccharide (dextran) from sucrose was studied. The enzyme was obtained from sucrose broth cultures of a strain of *Leuconostoc mesenteroides* by a method which yielded more potent preparations than were hitherto available.

2. Analyses of enzyme-sucrose mixtures for each of the known reactants showed that the conversion of sucrose to dextran proceeded nearly to completion according to the equation, n sucrose $\rightarrow$ dextran $+ n$ fructose.

3. The concentration of sucrose influenced the initial activity of the dextran-synthesizing enzyme in the same way as it does invertase. That is, the relationship between initial rates of dextran synthesis and sucrose concentrations below approximately 200 mM followed the expression of Michaelis and Menten, whereas velocities lower than the theoretical were obtained at higher sucrose concentrations. The Michaelis constant, K_s, calculated from the data at the lower sucrose concentrations, was approximately 19 to 20 mM.

4. The enzymatic synthesis of dextran simulated a first order reaction in systems containing 1 per cent sucrose but resembled a zero order reaction in systems containing 5 per cent sucrose. The corresponding unimolecular and zero order velocity constants can therefore be utilized to measure the activity of the enzyme.

5. The addition of purified dextran to enzyme-sucrose mixtures did not increase the initial velocity of dextran formation. (This failure does not, however, exclude dextran as an activating substance, because small amounts of the preformed polysaccharide, which might be sufficient to prime the reaction, regularly accompany the active enzyme.)

6. A number of well known enzyme inhibitors, including azide, cyanide, fluoride, and iodoacetate, were without effect upon the dextran-synthesizing enzyme.

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INTESTINAL AND LIVER FACTORS IN THE METABOLIC UTILIZATION OF HOMOCYSTINE*

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Experimental rats of this laboratory have consistently been found capable of an immediate growth response upon addition of homocystine to a synthetic diet free of other sulfur-containing amino acids and choline (2, 1), and biosynthesis of methionine under these conditions has been demonstrated (3). The fact that rats can grow without a known source of dietary N- or S-bound methyl groups is not in itself a new observation. Du Vigneaud *et al.* (4) in their pioneer investigations on labile methyl groups called attention to isolated animals which grew as much as 0.4 gm. per day for 8 weeks on their methyl-free homocystine diet, and considered the possibility that in these cases synthesis by intestinal organisms (termed refection at that time; *cf.* (5)) may have been involved. With the objective of elucidating the nature of the factors that may be responsible for the substantially greater independence of dietary methyl groups shown by our animals, we have previously studied the effect of certain changes in dietary vitamins and mode of food administration (2), with results which indicate that these factors are accessory determinants of growth response but do not explain the main divergence of our observations from those of du Vigneaud. Through the cooperation of Dr. du Vigneaud it became possible to experiment with a few rats of his strain, members of which had recently been found incapable of growth on a "choline-methionine-free diet containing homocystine."[1] The finding[2] that animals, bred under our animal colony conditions from parents of du Vigneaud's strain, proved capable of utilizing

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* Aided by a grant from the McNeil Laboratories, Inc., Philadelphia. A summary of parts of this work has been presented by Bennett (1).

[1] Personal communications of Dr. du Vigneaud and Dr. Chandler.

[2] Five female and two male rats, 5 to 6 weeks old, of du Vigneaud's strain transferred to our laboratory, were fed our colony diet but kept isolated from our stock rats in order to avoid direct bacterial transmissions. At the age of 3 months they were mated. Due to difficulty in breeding some of the offspring died, but eight animals were available for experimentation: four males and three females from one litter and one female, Rat 8, from another litter of different parents. When put on the choline, methionine-free diet plus eight B vitamins (at 5 weeks of age), addition of 1.25 per cent homocystine on the 8th day resulted in growth; all but one of the animals showed a 6 to 10 day gain in weight when first fed the homocystine, and after this period there was the usual loss of weight followed by resumption of growth in most animals. Two of the males and two females showed net gains of more than 25 gm. during a 70 day period: Rat 4 (male), 67 gm. and Rat 8 (female), 43 gm. Within the four male litter mates there was a substantial variation of growth: +67 gm. in 70

homocystine, even though less effectively than the animals of our own stock, strongly suggests that preexperimental nutrition is a determining agent in this phenomenon, especially since our colony diet surpasses the usual stock rations in nutritional value (2). This favorable preexperimental food supply could act by promoting nutritional storage in the animal body of factors required for subsequent assimilation of homocystine sulfur and also by enhancing development of intestinal organisms which in turn become the actual contributors of the essential factors. In order to examine the rôle of the second of these possibilities, a study was made of the effect, on the utilization of homocystine, of the inclusion in the diet of succinyl-sulfathiazole (known to be an inhibitor of intestinal synthesis of folic acid and other factors (6)) and its compensation by dietary accessories. The preliminary experiments of this study have been previously summarized (1); they indicated that certain growth interruptions consistently exhibited by some of our rats (2) are not related to a need for folic acid, as had been suspected, since this vitamin was not included in our regular vitamin supplement. They also showed that addition of succinylsulfathiazole to the homocystine diet stopped the growth of the animals in approximately 4 weeks, resulting in a situation (presumably due to changes in the intestinal flora) in which supplementation of the dietary vitamins with rice polishings extract permitted utilization of methionine, while homocystine could not be utilized. Utilization of homocystine became possible when a small amount of a certain liver fraction was fed. The liver fraction seemed to take the place of the intestinal factor eliminated or inhibited by the sulfonamide action. However, actual identity of the intestinal factor with a liver factor has not been demonstrated. A more detailed investigation of the nature of the liver effect is the object of the present work.

Diet and Analytical Data—The dietary components were of the same purity and from the same sources as before, and basal diet and vitamins were substantially identical[3] with those used previously (2). The *dl-*

days; +26 gm. in 70 days; +6 gm. in 60 days; −33 gm. in 14 days   The last two died showing ascitic fluid on autopsy; the others were killed. Autopsy findings and food consumption data were similar to those previously reported (2). During periods of acute weight loss daily food consumption dropped to zero, while in Rats 4 and 8 it rose to 7 and 6 gm.

[3] The percentage composition of the diet is amino acid mixture 17, *l*-lysine mono-hydrochloride monohydrate being substituted for *dl*-lysine monohydrochloride used in previous experiments (the former contained (7) 0.08 per cent methionine), dextrin 30, sucrose 17 (15 when 2 per cent sulfasuxidine was added), salt mixture 4 (Osborne and Mendel (8)), agar 2, and Mazola corn oil 30. Carotene (0.00028 per cent), tocopherol (0.00016 per cent), methylnaphthoquinone (0.00011 per cent), and vitamin D (6.2 U. S. P. units per gm.) are present in the diet, and the (separately administered) standard daily dose of B vitamins is 500 γ each of nicotinic acid, *p*-aminobenzoic acid, and inositol, 200 γ of calcium pantothenate, 40 γ each of thiamine hydrochloride, riboflavin, and pyridoxine hydrochloride, and 0.25 γ of biotin.

methionine used was du Pont's product ("99.93 per cent purity (perchloric titration)").

Sulfasuxidine (succinylsulfathiazole, Sharp and Dohme) was given as 2.0 per cent of the diet, replacing an equal amount of sucrose. Homocystine was also fed as a part of the diet, being added to the dry mixture in amounts corresponding to an extra 1.25 per cent or 0.83 per cent of the basal diet. Rice polishings extract was used as ryzamin-B or as ryzamin-B unfortified. The latter, except for its being approximately 20 per cent less concentrated, is the same product[4] as the former. The following liver products were used: liver extract Lilly, formerly called liver extract No. 343 and representing the anti-pernicious anemia Cohn fraction G (9), i.e., that part of aqueous liver extract that is soluble in 70 per cent ethanol but insoluble in 93 per cent ethanol; liver extract No. 55 (Lilly) representing the (protein) fraction insoluble in 70 per cent ethanol; desiccated liver extract No. 1 (Lilly), representing the fraction soluble in 93 per cent ethanol; solution liver extract parenteral Lederle, a product likewise representing the Cohn fraction G; and concentrated solution liver extract Lederle, a product derived from the preceding, but 4 to 5 times as high in antianemia potency. Available analytical data for these products are listed in Table I.

Animals—The general preexperimental diet containing a generous variety of natural foods has been previously described (2). During the experimental period each animal was housed in a separate cage, and, except for daily weighings, experimental conditions were the same as those outlined earlier[5] (15).

Twenty-four females from six litters were used. Twenty of the rats came from litters born within 3 days; the remaining four were approximately 18 to 22 days older. They were all placed on the sulfasuxidine-containing diet when the youngest were 34 days old.

First Experimental Series—The liver fraction previously used (1) was the same as that employed by White and Beach (16) in their experiment demonstrating utilization of homocystine for growth. The first objective was to determine the effectiveness of this liver product in quantitative terms, as a preliminary to its comparison with other related products.

In order to develop a deficiency of the unknown intestinal factor, the animals were directly transferred from the preexperimental diet to the sulfasuxidine-containing synthetic diet. Water-soluble vitamins were, as usual, given separately; they consisted of the usual eight B vitamin combination and, in addition, 62.5 mg., per animal per day, of ryzamin-B unfortified (product *t*, Table I). The latter was given with the intention

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[4] Personal communication of Dr. G. H. Hitchings of the Wellcome Research Laboratories.

[5] The rats drank only distilled water.

TABLE I

Analytical Data for Rice and Liver Products

	Total solids*	Total N	Choline N††	Total S‡§	Methionine S†§	Cystine S†‡	Sulfate S‡**	Humin S†	Thiamine*	Riboflavin*	Nicotinic acid*	Pantothenic acid*	Pyridoxine*	Anti-pernicious anemia potency U.S.P. units†† per gm. solids	
														Oral	Injectable
	mg. per cc.	per cent	per cent	per cent	per cent	per cent	per cent	per cent	γ per gm.	γ per gm.	γ per gm.	γ per gm.	γ per gm.		
(s) Ryzamin-B	Syrup, 25% more than (t)		0.096									400	100		
(t) " unfortified	Syrup	0.66†		0.080					240	25					
(u) Liver extract Lilly‡‡*	Powder	8.78†	0.128§	0.89	0.23	0.13	0.18	0.09	22	250	660	300	30	0.078	
(v) " " No. 55	"		0.005§	1.89	0.15	0.22	1.41		25	90	100	20			
(w) Desiccated liver fraction No. 1	"		0.185§	1.48	0.23	0.15	0.92		20	215	1465	309			
(y) Solution liver extract parenteral Lederle ‡‡	215.3	7.0*	0.415§		0.24	0.14	0.10								15
(z) Concentrated solution liver extract Lederle	74.0	8.6*													200

* According to information supplied by the manufacturers.
† According to our own determinations.
‡ Methods of Engel (13) and Glick (10).
§ For product t the method of Evans and St. John (11), for the others that of Callan and Toennies (12) was used.
‖ Method of Lavine (7).
¶ Method of Kassell and Brand (14).
** Gravimetrically in HCl hydrolysate.
†† An oral unit is 20 to 60 times (according to various sources of information) as large in amount as an injectable unit.
‡‡ Represents Cohn fraction G (9).
§§ There was no evidence (10) of the presence of betaine.

of maintaining the greatest possible degree of adequacy, apart from the specific requirements of homocystine utilization. After 8 days on the basal synthetic diet, the average weight of the twenty-four animals (ranging between 75 and 169 gm.) had fallen from 116 to 85 gm., and 1.25 per cent homocystine was added to the diet. After 13 days of this regimen the average weight was 92 gm., reflecting the growth-promoting effect of homocystine. Presumably growth during this period would have been larger, had not the consumption of food been restricted to 3 and (for the larger animals) 4 gm. per day. The restriction was imposed in the hope of minimizing the usual growth crises and the risk of losing animals as a consequence of overconsumption of homocystine. Similar considerations prompted a lowering of the homocystine content of the diet, after the first 13 days, to 0.83 per cent, with continued restriction of intake. After 7 days under these conditions (*i.e.* a total of 4 weeks of sulfonamide diet) the average weight was 95 gm. In order to see whether by this time the sulfasuxidine had taken effect the rats were allowed to eat *ad libitum* for 1 day. They responded to the *ad libitum* feeding by an average overnight consumption of 4.3 gm. and gains averaging 2.1 gm. Food consumption was again restricted and, because of the possibility that continued ingestion of ryzamin might somehow hinder the action of sulfasuxidine on the intestinal flora, the rice extract was withdrawn from the daily vitamin supplement. During the next 13 days the average weight changed from 97 to 88 gm. However, during (in a few cases shortly before and after) this period about one-half of the animals experienced the characteristic growth interruption crisis; in eleven animals the average consecutive loss was 20 gm. and the average duration 5 days. While these drops were similar in symptoms and extent (except for their greater rapidity) to those previously recorded under different conditions ((2) p. 69), recovery was stunted, presumably reflecting the existence, by this time, of permanent deficiencies in intestinal biosynthesis; the amount of weight regained averaged only 5 gm. during the 10 days following the lowest point. The ryzamin supplement was now reestablished, the biotin content of the daily supplement was increased by 2 γ, and the animals were allowed to eat *ad libitum*. The growth patterns ensuing during the next week (the 7th of sulfonamide ingestion) suggested that the effect of the drug was now fully established.[6] Average growth and food data of this period are included in Fig. 1. The rats were now arranged into experimental groups. For the quantitative assay of the liver extract the twenty most suitable animals were divided into five groups of four rats each, equalizing them as well as possible with regard to average weight during the immediately preceding 10 days, as

[6] What effect, if any, the temporary withdrawal of the rice polishings extract has had in the attainment of this state cannot be deduced from this experiment.
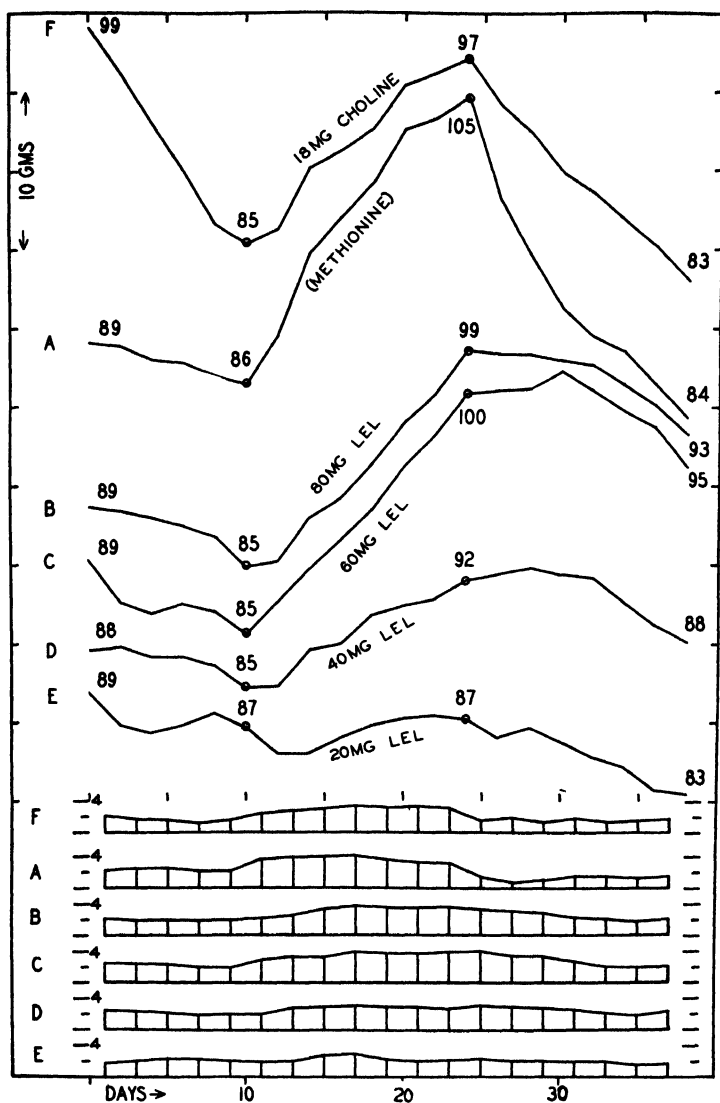
FIG. 1. Average growth and food consumption in the first experimental series. The diet, arrangement of groups, dietary changes, and supplements are given in detail in the text.   Average animal weights of the groups are plotted every 2nd day, and daily food consumption is shown as the average of consecutive 2 day periods. The numerals show the average weights 10 days preceding the experimental period and at the subsequent points of dietary change.   Methionine indicates the substitution of methionine for homocystine in the dietary mixture.   "LEL" stands for liver extract Lilly.

well as with regard to their ·previous history, each group receiving two·
animals that had experienced a period of acute weight loss.   Four of these
(Groups B, C, D, E) were continued on their current diet, supplemented,
however, by daily doses (given by pipette as an aqueous suspension) of
80, 60, 40, and 20 mg. respectively of liver extract Lilly.   The fifth
(Group A) received no extra supplement, but the dietary homocystine was
replaced by an equimolar amount of *dl*-methionine.   The four rats re-
maining after selection of these groups were those rejected because they
still appeared to be in various stages of acute growth regression.   This
group (F) was utilized for the purpose of testing the effect of choline which
had not previously been determined on a sulfonamide-containing diet.
The base was given with butter, as previously described (2), in daily
amounts of 18 mg.   It should be noted that from a quantitative viewpoint
the response of this group is probably not comparable with that of the
others.   The dietary changes just outlined were maintained for 14 days
and followed by a return, for 14 days, to the preceding diet.   Fig. 1 shows
the effects of these variations.   The average growth rates during the supple-
ment periods (the first 2 days of each being disregarded) are 0.23, 0.56,
1.07, 1.10, and 1.24 gm. per day (the lowest and highest rate within each
group being 0.00, 0.31; 0.35, 0.84; 0.96, 1.40; 0.75, 1.44; 0.75, 2.00) re-
spectively, on homocystine with 20, 40, 60, and 80 mg. of liver extract and
on methionine instead of homocystine.   Average daily food consumption
in these groups was 2.48, 2.90, 3.62, 3.36, and 3.46 gm.

Discussion—It would appear, in terms of resultant growth, that daily
ingestion of 0.22 milliatom of homocystine sulfur (30 mg. of homocystine)
in combination with 60 mg. of liver extract (containing 0.38 milliatom of N,
including 0.0051 mM or 0.62 mg. of choline, 0.013 milliatom of organic S,
including 0.0043 mM or 0.64 mg. of methionine) is only slightly inferior to
an equal amount of methionine sulfur (32 mg. of methionine).   The data
of Medes, Floyd, and Cammaroti (3) indicate that in an animal growing 1.1
gm. per day about 7 mg. or 0.045 mM of methionine is deposited daily in
new tissue, and the actual minimum metabolic requirement of labile methyl
is likely to be about 3 times as high ((2), foot-note p. 80).[7]

[7] Under the prevailing experimental conditions the daily amount of dietary
methionine required for mere maintenance of weight is higher than 7 mg., as in-
dicated by the following.   After completion of the scheduled experiments several
of the rats were placed on a diet which, instead of 0.83 per cent homocystine, con-
tained 0.23 per cent (one-quarter of the molar equivalent) of methionine, with all
other conditions unchanged.   Five animals, weighing 93 to 110 gm., lost between
1.4 and 5.6 gm. during 8 days on this diet; food consumption was limited to 3 gm. per
day but on several days remained actually below this level.   Two of the rats per-
mitted to eat *ad libitum* for an additional 8 days lost 0.6 and 3.5 gm. respectively,
on average daily food consumption of 3.0 and 2.4 gm.
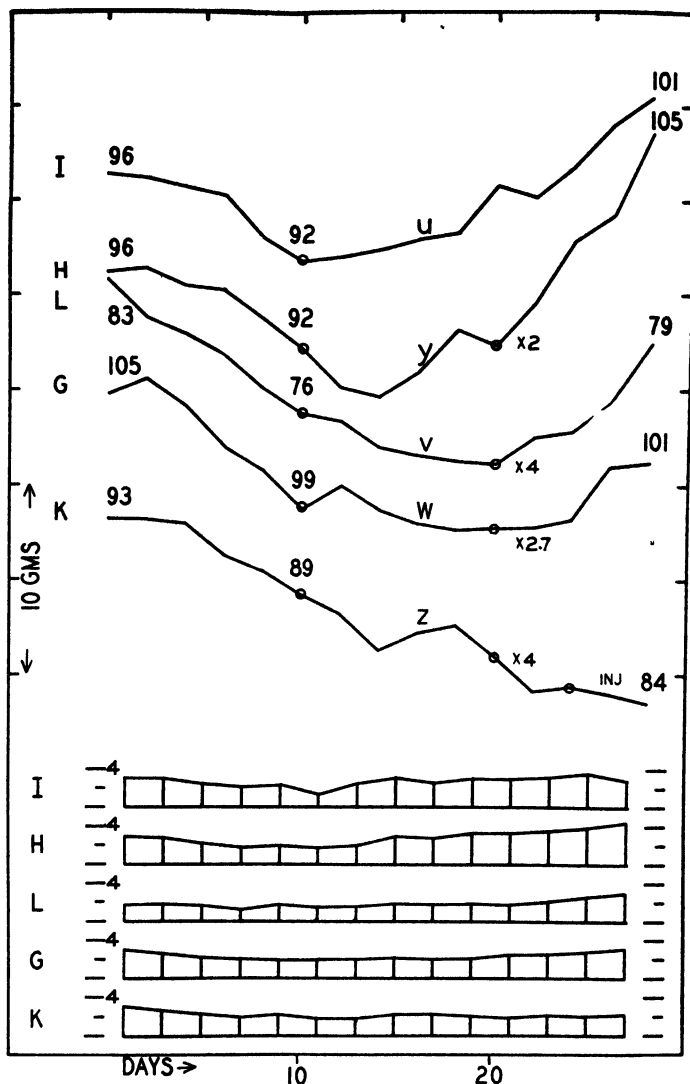
, FIG. 2. Average growth and food consumption in the second experimental series. The method of plotting is the same as in Fig. 1; the small letters refer to Table I (see the text). The following supplements (*cf.* "Diet and analytical data") and daily amounts were given (orally unless otherwise stated): Group I, 40 mg. of liver extract Lilly (product *u*); in this group one rat had to be eliminated during the test period because of obvious symptoms of illness (listlessness and abdominal distension). This group received the same treatment as that represented by "40 mg. LEL" (Curve D), Fig. 1. Group H received 0.235 cc. of solution liver extract parenteral Lederle (*y*); *i.e.*, 51 mg. of dry substance equivalent in N content to 40 mg. of liver extract Lilly (*u*); after 10 days the dose was doubled ("×2"). Group L was

The immediate and sharp weight losses resulting from replacement of methionine by homocystine, as compared with the delayed and slow declines following withdrawal of liver extract, are noteworthy, but pending further knowledge of the liver factor involved, need not be discussed. The fact that with the restriction in vitamin resources caused by the dietary sulfonamide, and only partially compensated by the rice extract, choline is as effective in eliciting utilization of homocystine as is the liver extract indicates that the active factor in the latter is not one involved in the mobilization of the methyl groups of dietary choline (transmethylation).

Second Experimental Series—The purpose of this series was to compare a number of different liver products with regard to the presence of the unknown factor and to ascertain its relation to the antianemia principle (since the liver fraction used in the preceding experiments is the one used for the treatment of pernicious anemia). Rather than repeat the long and costly process of conditioning fresh rats, the test subjects for this phase of the work were recruited from the animals used in the preceding assay. They were now 16 to 19 weeks old, had been on sulfonamide-containing diets continuously for 11 weeks, and their average weight was 88 gm.; *i.e.*, 3 gm. higher than at the beginning of homocystine supplementation 10 weeks earlier. For the last 2 weeks all had been fed alike (basal diet with homocystine, eight B̆ vitamins, including extra biotin and ryzamin). Four new groups of three animals each were selected from the three previous groups that had been on supplements of 80, 60, and 40 mg. of liver extract, in such a manner that each new group contained one rat from each of the three previous groups. For the fifth group of three, one animal from each of the previous choline, methionine, and 20 mg. liver extract groups was chosen. The resulting new group was used for the testing of the liver protein fraction (product *v*, Table I). The results obtained in the comparison of five liver products are summarized in Fig. 2, and details concerning modes of administration and doses are indicated in the legend. Comparison was based on a suboptimum dose (40 mg.) of the reference standard (liver extract Lilly) to permit disclosure of higher concentrations of the active principle in any of the other products.

Discussion—The results indicate that the Cohn fraction G dispensed as a solution (product *y*, Table I) has a similar, though somewhat weaker,

given 40 mg. of liver extract No. 55 (*v*); after 10 days the dose was increased by 300 per cent ("×4"). Group G received 40 mg. of desiccated liver fraction No. 1 (*w*); after 10 days the dose was increased by 170 per cent ("×2.7"). Group K was fed 0.051 cc. of concentrated solution liver extract Lederle (*z*), *i.e.* 3.8 mg. of dry substance, equivalent in injectable antianemia units to the initial supplement of Group H; after 10 days the dose was increased by 300 per cent ("×4"). For the last 4 days the initial dose (0.05 cc.) was given by intramuscular injection ("INJ").

activity than the solid Cohn fraction G (u), when compared on the basis of N content.   The fractions less (v) and more (w) soluble in alcohol than the Cohn fraction G seem to contain the active agent in small concentrations. Thus its distribution within the alcohol-precipitated fractions runs parallel to that of the antianemia agent.   However, the possibility of the two agents being identical appears to be ruled out by the completely negative results of the antianemia concentrate (z).   In terms of physiologically available antianemia units, the dose injected during the last 4 days of the latter experiment should be at least 10 times as potent (cf. foot-note, Table I) as the oral dose received during the same period by Group H. That intramuscular injection represents an effective route of administra- of the homocystine activator was established by a supplementary experi- ment.   A rat, after having been transferred from a methionine-containing diet to homocystine, lost 4.3 gm. in 4 days; upon daily injection of 0.35 cc. of the parenteral liver extract (y) it gained 9.0 gm. during the next 2 weeks. Upon discontinuance of the injections the net change for 2 weeks was zero, followed by a loss of 10 gm. during another 2 weeks.

Further characterization of the liver factor effective in homocystine utilization is needed, and, except for the immediate response to intra- muscular injection, there is nothing in the present evidence which is at variance with the hypothesis that the growth requirements of the rat may be satisfied by methyl compounds elaborated by intestinal symbionts and that the availability of such extraneous methyl groups is conditioned, among other factors, by the preexperimental diet (cf. (17)) and by certain vitamin-like dietary factors which are not needed when the methyl re- quirements are satisfied by methionine or choline.

SUMMARY

It is shown that the capacity of rats to grow on a homocystine-containing diet devoid of other sulfur-containing amino acids and known methyl

donors is not a phenomenon limited to a single strain of animals. Experiments with another strain of rats indicate that the capacity depends on internal factors arising from preexperimental nutritional conditions. Modification of intestinal flora by sulfonamide action produces a situation in which supplementation of the dietary vitamins with rice polishings extract permits utilization of methionine, while homocystine cannot be utilized. Utilization of homocystine becomes possible when either choline is fed or small amounts of certain liver fractions. Analytical data show that methionine, choline, or betaine is not their effective component, and comparison of different products indicates that it is not identical with the antianemia principle of liver, although its distribution parallels that of the latter to some degree.

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THE INFLUENCE OF PREEXPERIMENTAL FOOD ON THE UTILIZATION OF HOMOCYSTINE IN A "METHYL-FREE" DIET*

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It has been suggested in the previous paper (1) that preexperimental diet may play an important rôle in the ability of the rat to utilize homocystine on a "methyl-free"[1] diet. Rats from another laboratory, which would not grow on the above diet, acquired definite ability to utilize it when fed our preexperimental colony diet for two generations. This colony food, which is of high standard, could influence favorably the liver storage of the rats and also their intestinal flora. The importance of the latter becomes evident if bacterial synthesis of unknown factors influencing homocystine utilization is involved.

Results of the experiments conducted in this laboratory with a methyl-free homocystine diet containing 2 per cent sulfasuxidine (2, 1), suggest that bacterial synthesis does play a rôle in methylation under conditions of dietary labile methyl deficiency. A recent report by du Vigneaud et al. (3) gives evidence, by means of D_2O, of the synthesis of small, but not insignificant amounts of labile methyl groups by the white rat on a diet adequate in these groups (20 per cent casein). Intestinal bacterial synthesis is suggested as the most logical interpretation of their results.

Six rats, from our previous set of experiments (1), that had lost their ability to utilize homocystine, due to having lived 5 months on a 2 per cent sulfasuxidine methyl-free diet, were available for further study. These rats had experienced short periods of growth, due to the fact that they had received labile methyl groups and liver extract in their diets at intervals during the course of the experiment; but whenever these were withdrawn and homocystine alone was present, they ceased to grow. It seemed worth while to find out whether these animals could regain their ability to grow on a methyl-free homocystine diet if put back on the colony food for several weeks.

[1] The term "methyl-free" is used to designate the absence of known dietary methyl donors.
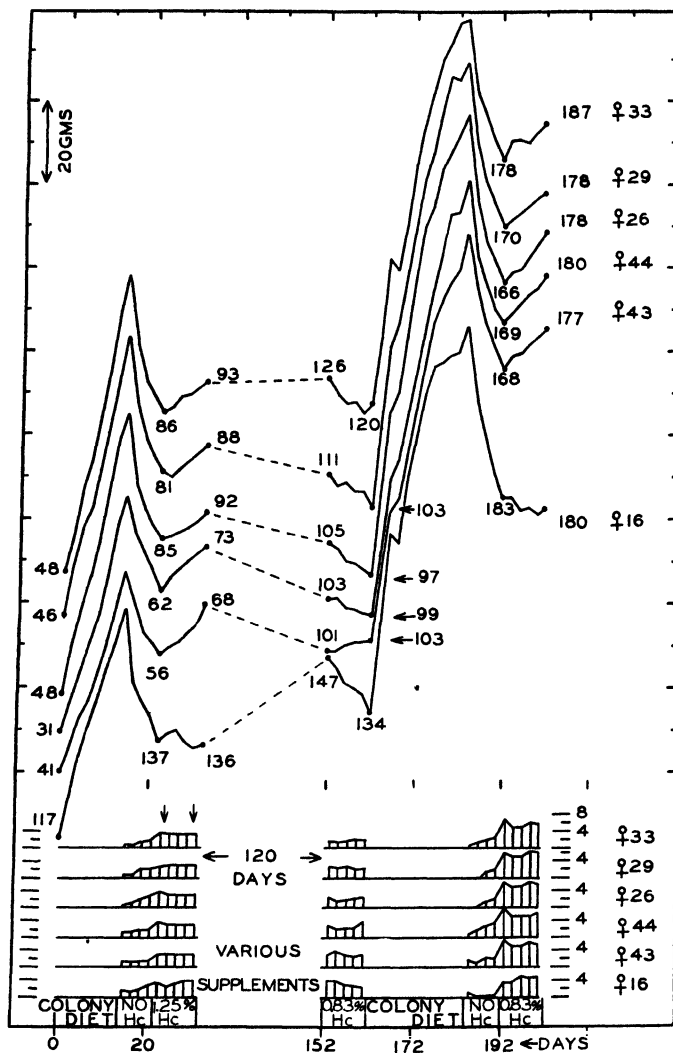
FIG. 1. Growth and food consumption curves. The weight figures are plotted every other day and daily basal food consumption is shown as the average of consecutive 2 day periods. "Hc" designates homocystine; the arrows over the food curves for the 1.25 per cent homocystine period indicate that the diet was restricted (see the text).

Diets

The colony diet contains liver, eggs, fish, cheese, peanuts, yeast, carrots, lettuce, tomatoes, oranges, cabbage, oats, checkers (Purina), milk with navitol and zygon (Squibb), bone meal, and salt rings (4).

The experimental diet was that described in the previous paper (1).

Rats 26 and 29; 33; 43 and 44 were born within 3 days of each other and represent three litters; they were started on the basal diet at approximately 5 weeks, Rat 16 from a fourth litter at 7 weeks of age (cf. Fig. 1). The initial weights are given 2 weeks before starting the basal diet, at which time the rats were eating colony food. At the end of these 2 weeks they were depleted for 8 days on the methyl-free basal diet containing 2 per cent sulfasuxidine; 1.25 per cent homocystine was then added and after the first day the diet was restricted to 3 gm. (4 gm. for Rat 16) in order to prevent toxicity due to overeating (cf. (1)). The curves for this 10 day period demonstrate the usual response of our rats to homocystine: five grew, Rat 16, for some unknown reason, being an exception; at this stage the sulfasuxidine had not had time to become effective. The next 120 days are omitted, as they pertain to the previous paper. At the end of the 120 day period when the rats had been on sulfonamide for 138 days, a final 10 day period is recorded showing the response of these rats to an ad libitum 0.83 per cent homocystine diet containing 2 per cent sulfasuxidine. It is evident that they had lost their ability to utilize homocystine, and their food consumption had definitely dropped. The animals kept in the same cages were then put back on the original colony food (no sulfasuxidine) for 3 weeks,[2] depleted as before for 8 days, then fed ad libitum the same 0.83 per cent homocystine diet containing 2 per cent sulfasuxidine. Five rats responded to this diet and grew at practically the same rate as they had on the previous (restricted) 1.25 per cent homocystine regimen; their food consumption greatly increased. The fact that Rat 16 failed again to respond to a homocystine diet indicates that a factor of individual constitution also plays a rôle. After this 10 day period the rats were killed, an autopsy performed, and histopathological examinations were made of the livers and kidneys; the results are reported below (cf. (4)).

Histopathology

The livers of all six rats showed severe parenchymatous and fatty degeneration; the severity of these symptoms was most pronounced in Rats 44 and 16. Rat 33 showed, in addition, slight early cirrhosis.

The kidneys of all six rats showed general degeneration with the major change in the tubules; there was also some hemorrhage. Rats 44 and 16 manifest the most severe pathological changes.

[2] It is of interest to note that apparently the rats retained their ability to hydrolyze protein during 5 months on an amino acid mixture, as evidenced by their growth response when returned to the colony diet.

SUMMARY

Rats which could utilize homocystine for growth on a "methyl-free" diet lost this ability after having lived 5 months on the same diet plus 2 per cent sulfasuxidine. However, this capacity was· regained after the animals had been put back on the preexperimental colony food for an interval of 3 weeks. These results seem to add evidence in support of the theory of bacterial synthesis of unknown factors influencing homocystine utilization; at least, they demonstrate that the mechanism involved can be reestablished by a return to our colony diet.

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THE ANTIINOSITOL EFFECT OF γ-HEXACHLOROCYCLO-HEXANE*

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It has been reported by Slade (1) that the insecticidal action of the mixture of the four hexachlorocyclohexanes forming the insecticide "666" is almost entirely due to the γ isomer. Further, Mooney (1) has pointed out the resemblance between the hexachlorocyclohexanes and the cyclohexitols. Slade states that the English workers have presumptive evidence that the γ isomer has the same spatial configuration as *i*-inositol. Their evidence was based upon infra-red spectroscopy and the relative probability of the formation of each isomer as compared with the actual occurrence of the isomers in the crude preparation of hexachlorocyclohexanes. They postulated further that the insecticidal action of the active isomer might be due to its interference with the inositol metabolism of the insect.

Dacey, Colucci, and Kirkwood[1] attempted to determine the spatial configurations of the hexachlorocyclohexanes. All attempts to convert the isomers to cyclohexitols, whose configurations could be determined by the method of Dangschat and Fischer (2), were unsuccessful. Biological evidence is now available which indicates that the insecticidal γ isomer has the same configuration as *i*-inositol. The purpose of this report is to present data which show that γ-hexachlorocyclohexane inhibits the growth of a yeast which requires an exogenous source of inositol and that the effect of the γ isomer is reversible upon the addition of sufficient *i*-inositol.

EXPERIMENTAL

The approach to this problem seemed possible through the use of an inositol-requiring organism, an inositol-free medium, and the administration of graded concentrates of the cyclohexanes and of *i*-inositol.

The Gebrüder Mayer strain of *Saccharomyces cerevisiae* was chosen as the test organism. The medium employed was that of Williams *et al.* (3) modified by the omission of the calf liver supplement and the inclusion

of the non-dialyzable portion of 5 gm. of rice bran extract (vitab) per liter. Inocula were grown for 24 hours on the basal medium supplemented with 2 γ of i-inositol per ml. The cells were collected by centrifugation, washed three times with the basal medium, and suspended in sufficient basal medium to give a reading of 85 on the Evelyn colorimeter, with a No. 660 filter, on which the reading for the basal medium is 100. The inoculum consisted of 1 drop of this cell suspension. The incubation period in all cases was 18 hours at 30°.
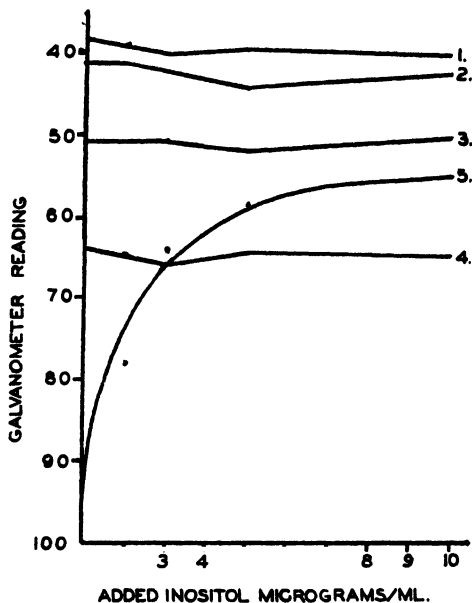
FIG. 1. The effect of added i-inositol on the growth of the Gebrüder Mayer strain of yeast in media containing hexachlorocyclohexanes. Curve 1, control; Curve 2, 20 γ of β isomer per ml.; Curve 3, 40 γ of α isomer per ml.; Curve 4, 50 γ of δ isomer per ml.; Curve 5, 60 γ of γ isomer per ml.

Assay Procedure—To a 40 ml. portion of the basal medium containing the desired level of inositol was added 1 ml. of an ethyl alcohol solution of the appropriate isomer. The alcohol solution of the isomer was of such a concentration that it gave the desired test concentration in the 40 ml. of medium (20 to 60 γ per ml.). Three 11 ml. aliquots of this medium were placed in 50 ml. Erlenmeyer flasks which were sterilized in an autoclave (15 pounds for 15 minutes), inoculated, and incubated as described. The contents of each flask were then examined quantitatively for turbidity in an Evelyn colorimeter with a No. 660 filter.

Preparation of Isomers—A mixture of the hexachlorocyclohexanes was prepared by passing dry chlorine gas through benzene refluxing in a Pyrex

flask irradiated by a mercury vapor lamp. The crude mixture of isomers readily crystallized when the excess benzene was removed under reduced pressure. The four isomers were separated by the procedure of van der Linden (4). The melting points of the preparations agree well with those reported by the English workers; *i.e.*, α 158°, β 309°, γ 112°, and δ 139°.

Results

The results are shown in Fig. 1. Growth, as indicated by turbidity, was plotted against micrograms of purified *i*-inositol added to the media.[2] The Gebrüder Mayer strain of yeast requires 1 γ of *i*-inositol per ml. for maximal growth: The α, β, and δ isomers at the limit of their solubility in the medium, that is 40, 20, and 50 γ per ml. respectively, inhibited the growth of the Gebrüder Mayer yeast. The inhibition produced by α and β isomers was comparatively slight, while that produced by δ was more pronounced. Slade (1) has reported these compounds to be relatively inactive as insecticidal agents. However, in all three cases the inhibition was not affected by the addition of inositol. The γ isomer markedly inhibited growth of the yeast at the limit of its solubility (60 γ per ml.). This inhibition was progressively but not completely reversed by the addition of from 1 to 6 γ of *i*-inositol per ml. of medium. A strain of yeast which did not require an exogenous source of inositol was only slightly inhibited by the γ isomer.

DISCUSSION

The fact that the inhibition of Gebrüder Mayer yeast growth by γ-hexachlorocyclohexane was reversed by *i*-inositol offers excellent biological evidence that the γ isomer has the same spatial configuration as *i*-inositol. Evidence is accumulating in the literature which points to the close structural similarity between mutually antagonistic substances. This subject has been ably reviewed by Welch (5). It is assumed in this case that γ-hexachlorocyclohexane inhibits inositol metabolism by means of the spatial configuration of its chlorine atoms, which enables it to block effectively the normal function of *i*-inositol. Further, the low molecular inhibition ratio[3] $c_i/c_m = 30$ (McIlwain (6)) in this case would indicate a very close structural similarity to *i*-inositol. The ratio would indicate that the chlorine atoms in the isomer are arranged spatially above and below the ring in the same manner as are the hydroxyl groups in *i*-inositol (Dangs-

[2] The *i*-inositol used in this experiment was graciously supplied by Dr. H. A. Lardy. It was prepared from recrystallized hexapropionylinositol by catalytic saponification.

[3] c_i is the molar concentration of inhibitor necessary to prevent the growth of an organism in the presence of a molar concentration of metabolite, c_m.

chat and Fischer (2)).   These data lend support to the hypothesis of the English workers (1) that the γ isomer exerts its insecticidal action by interfering with the inosital metabolism of the insect.

SUMMARY

γ-Hexachlorocyclohexane inhibited the growth of the Gebrüder Mayer strain of *Saccharomyces cerevisiae*.   This inhibition was found to be reversible by the addition of *i*-inositol.

The other three known hexachlorocyclohexanes inhibited the growth of Gebrüder Mayer yeast to a slight degree but this inhibition was not reversible by *i*-inositol.   These data correlate closely with their demonstrated ineffectiveness as insecticidal agents.

These data suggest that the spatial configuration of the γ isomer is similar to that of *i*-inositol and further support the hypothesis that the γ isomer exerts its insecticidal action by interfering with the inositol metabolism of the insect.

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THE ACTIVITY OF "LACTOBACILLUS CASEI FACTOR," "FOLIC ACID," AND "VITAMIN B$_c$" FOR STREPTOCOCCUS FAECALIS AND LACTOBACILLUS CASEI

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It has been established that the terms vitamin M, vitamin B$_c$, norit eluate factor, *Lactobacillus casei* factor, factor U, folic acid, and factor SLR all refer to a group of compounds (or a compound) with similar characteristics and similar biological activities. Of these factors seven have been reported in purified form: folic acid (isolated from spinach) (1), three *L. casei* factors (one from liver, one from yeast, and one from a fermentation residue) (2, 3), synthetic liver *L. casei* factor (4), vitamin B$_c$ (from liver or yeast) (5), and factor SLR (6).

Pure folic acid was originally (1) assigned a potency of 40,000 (*i.e.* the pure compound was said to be 40,000 times as active as Wilson's liver fraction B). In the work reported from this laboratory (7) on the excretion of folic acid in sweat and urine, a folic acid concentrate furnished by Dr. R. J. Williams was used as standard, and the results were reported in terms of 40,000 potency. However, other active compounds have become available in pure form and Mitchell, Snell, and Williams (8) have reported a folic acid concentrate with a potency of 137,000. Therefore, in order to obtain a means of comparing data reported on the basis of 40,000 potency folic acid with data based on pure compounds as standards, the relative activities of vitamin B$_c$, folic acid concentrate, synthetic *Lactobacillus casei* factor, and fermentation *L. casei* factor for *Streptococcus faecalis* and *L. casei* were determined. The activities of several of these materials in terms of requirements for half maximum growth have been reviewed by Luckey, Teply, and Elvehjem (9) and by Peterson and Peterson (10).

EXPERIMENTAL

Streptococcus faecalis Assay—*Streptococcus faecalis* folic acid assays were carried out by the procedure of Luckey, Briggs, and Elvehjem (11) modified by the inclusion of 5 cc. of Salts B (12) and the replacement of pyridoxine by 100 γ of pyridoxamine per 500 cc. of double strength medium. Turbidities were read at 650 mμ with the Coleman universal photoelectric colorimeter after 16 hours incubation at 30°.

The activities of the following materials were compared: (a) a 7.7 per cent folic acid concentrate of potency 3100 (1) kindly supplied by, Dr. R. J. Williams of the University of Texas, (b) a solution of crystalline vitamin B$_c$ (5) kindly furnished by Dr. O. D. Bird of Parke, Davis and Company, (c) a solution of crystalline *Lactobacillus casei* factor (3) and

•—TEXAS "FOLIC ACID" CONCENTRATE (CALCULATED AT 137,000 POTENCY)
o—LEDERLE SYNTHETIC "L. CASEI FACTOR"
x—VITAMIN B c (PARKE-DAVIS)
+—TEXAS "FOLIC ACID" CONCENTRATE (CALCULATED AT 40,000 POTENCY)
*—LEDERLE CRYSTALLINE "FERM. L. CASEI FACTOR"
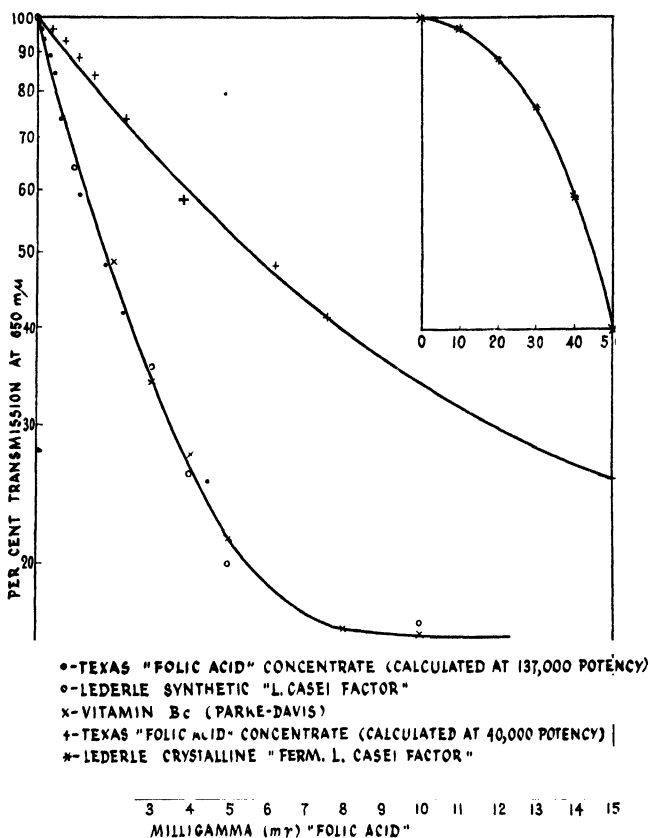
FIG. 1. Comparison of activity of different "folic acid" standards for *Streptococcus faecalis.*

(d) synthetic *Lactobacillus casei* factor (4) both kindly supplied by Dr. E. L. R. Stokstad of the Lederle Laboratories, Inc.

The dose-response curves for these compounds on the growth of *Streptococcus faecalis* are given in Fig. 1. The folic acid is plotted both on the basis of 40,000 potency as usual and also on the basis of 137,000 potency.

Lactobacillus casei Assay—Two methods of assay were used: the Landy and Dicken method (13) modified by the addition of 10 γ of *p*-aminobenzoic

acid and the replacement of the pyridoxine by 100 γ of pyridoxal per 500 cc. of double strength medium, and the method of Teply and Elvehjem (14). With both methods the tubes were titrated after 72 hours incubation at 37°.

The same materials were compared as to relative activity for *Lactobacillus casei* as had been tested with *Streptococcus faecalis* and the dose-response curves are plotted in Fig. 2. For these assays the medium of Teply and Elvehjem was used. While the relative activities of the four standards were the same by the modified Landy and Dicken medium, the total
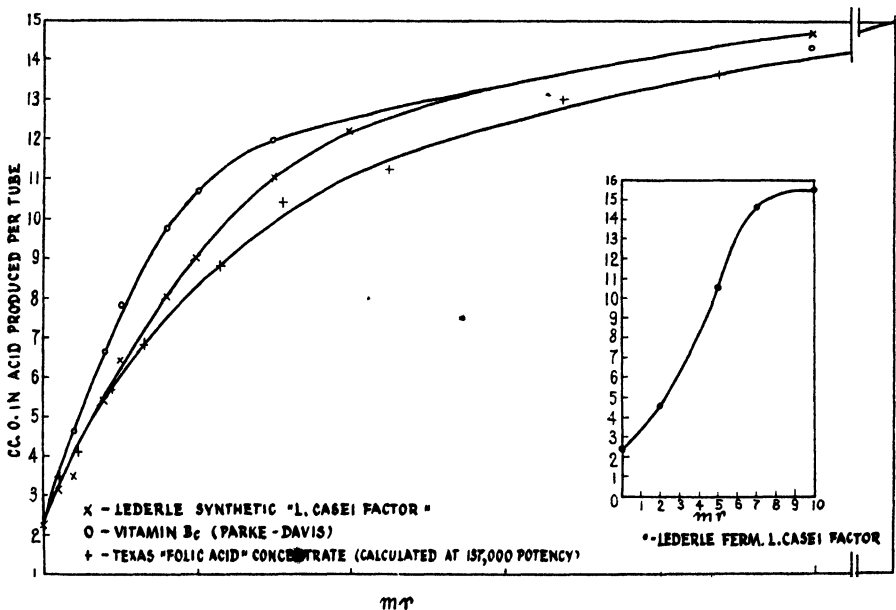
FIG. 2. Comparison of activity of different "folic acid" standards for *Lactobacillus casei*.

amount of acid produced was much less. In Fig. 2 the Texas folic acid concentrate is plotted on the basis of 137,000 potency only.

DISCUSSION

From Fig. 1 it is evident that Lederle synthetic *Lactobacillus casei* factor, Parke-Davis vitamin B_c, and Texas folic acid, calculated on the basis of 137,000 potency for the pure material, have equal activities for *Streptococcus faecalis*. Lederle fermentation *Lactobacillus casei* factor has only one-twentieth of the potency of these other materials. These data indicate that the first three materials are the same compound.

Some differences in activity are indicated in Fig. 2. However, in view of the lack of difference in response with *Streptococcus faecalis*, these differences may not be significant. These curves were obtained with the Teply and Elvehjem (14) medium with 1 mg. of norit-treated peptone per tube. The differences in the graphs may represent the effect of traces of other materials present rather than different compounds possessing different activities.

The half maximum doses for the compounds calculated from Fig. 2 are vitamin B_c 0.50 millimicrogram per tube, Lederle synthetic *Lactobacillus casei* factor 0.70 millimicrogram per tube, and Texas folic acid (137,000 potency) 0.80 millimicrogram per tube and *L. casei* factor 3.7 millimicrograms per tube. These figures agree favorably with those summarized by Luckey, Teply, and Elvehjem and by Peterson and Peterson (10). It was found that by changing the amount of norit-treated peptone in the Teply and Elvehjem medium (14) these half maximum doses could be changed. Eliminating the peptone increased the half maximum dose for Texas folic acid from 0.80 millimicrogram to 1.0 millimicrogram per tube, and increasing the peptone from 1 mg. to 2 mg. per tube decreased the half maximum dose for vitamin B_c from 0.50 millimicrogram to 0.35 millimicrogram per tube. The inverted dose-response curves to the crystalline *L. casei* factor (from a fermentation residue) was reported previously (7) and has recently been confirmed by Dr. E. L. R. Stokstad.[1] Day *et al.* (15) have reported that treatment of a similar preparation with enzyme greatly increased its activity toward *Streptococcus faecalis*.

Because three of the standard materials had equal activities for *Streptococcus faecalis* and *Lactobacillus casei*, it was decided to compare the activities of other materials for these two organisms, following enzymatic digestion. The values obtained for Difco yeast extract were 84 γ per gm. for *Lactobacillus casei* and 83.5 γ per gm. for *Streptococcus faecalis*. The enzyme used was desiccated hog kidney prepared by the method of Bird *et al.* (16). Other materials assayed did not give similar values with the two organisms. This may be due either to incomplete enzymatic liberation of the conjugates present in the other materials or merely to different relative activities of related compounds, as has been found for biotin and its vitamers (17), pyridoxine and its metabolites (18), and nicotinic acid and nicotinamide (19).

SUMMARY

1. A comparison was made of the activities of four different "folic acid"-active materials: synthetic *"Lactobacillus casei* factor" (Lederle), *"L. casei* factor" (Lederle), "vitamin B_c" (Parke-Davis), and "folic acid" concentrate (Texas) as growth factors for *Streptococcus faecalis* and *L. casei*.

[1] Private communication.

2. The synthetic *"Lactobacillus casei* factor," "vitamin B_c," and "folic acid" calculated on the basis of 137,000 potency for pure "folic acid" were found to be equally active for *Streptococcus faecalis* and almost equally active for *Lactobacillus casei*, with "vitamin B_c" exhibiting the greatest activity and "folic acid" the least.

3. Yeast extract alone gave equal assay values with both organisms when various materials were assayed following digestion with "vitamin B_c conjugase."

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THE MECHANISM OF IN VITRO AND IN VIVO INHIBITION OF CHOLINESTERASE ACTIVITY BY DIISOPROPYL FLUOROPHOSPHATE

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The alkyl fluorophosphates were described by Lange and Krueger (1) in 1932. Of this series of compounds, several have been studied in detail because of their marked toxicity. One of these compounds is diisopropyl fluorophosphate, a clear colorless liquid, which is only slightly soluble in water and soluble in organic solvents. The similarity between the

$$C_3H_7O \quad \diagdown \quad O$$
$$P$$
$$C_3H_7O \diagup \quad \diagdown F$$
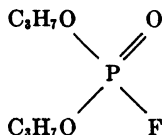
cholinergic effects of the fluorophosphates and those of physostigmine was noted by the British workers, McCombie *et al.*,[1] and by Adrian and his group.[2] The theory of chemical mediation of the transmission of nerve impulses through the autonomic nervous system identifies acetylcholine as the mediator. The presence of the enzyme, cholinesterase, at sites where acetylcholine is liberated by the nerve impulse leads to the rapid hydrolysis of acetylcholine. Since physostigmine decreases the activity of cholinesterase *in vitro*, it is presumed that the acetylcholine-like action of physostigmine is due to a similar enzyme inhibition *in vivo*, with resultant accumulation of acetylcholine. Because of the physiological similarity of diisopropyl fluorophosphate and physostigmine, Mackworth[3] studied the effect of incubating alkyl fluorophosphates with horse serum cholinesterase. He observed inactivation of the enzyme.

The present work is concerned with a study of the mechanisms of the *in vitro* and *in vivo* inhibition of the cholinesterase activities of serum, red blood cells, and tissues in the rabbit, monkey, and man by diisopropyl fluorophosphate.

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[1] McCombie, H., Adrian, E. D., Kilby, B. A., and Kilby, M., personal communication (1941). ✓

[2] Adrian, E. D., Kilby, B. A., and Kilby, M., personal communication (1942).

[3] Mackworth, J. F., personal communication (1942).

The method for the determination of cholinesterase activity was essentially that of Ammon (2). The production of acetic acid from acetylcholine was followed at 38° by means of the liberation of carbon dioxide from a bicarbonate-carbonic acid buffer in Warburg vessels. In the final reaction mixture of 4 cc., the concentration of acetylcholine was 0.015 M, except when otherwise stated; that of the $NaHCO_3$ was 0.04 M (pH 7.7) and, in some earlier experiments, 0.025 M (pH 7.5). The vessels were gassed at 38° with a mixture of 95 per cent nitrogen and 5 per cent carbon dioxide. At zero time, the enzyme preparation in the side arm was tipped into the acetylcholine-bicarbonate solution in the main compartment of the vessel, and readings begun 10 minutes after tipping.

The various enzyme sources studied were those of serum or plasma, red blood cells, muscle, and brain of the rabbit, rhesus monkey, and man. Rabbit serum was used undiluted; monkey and human sera or plasmas were diluted 1:5 with 0.025 M $NaHCO_3$. Red cells were centrifuged and washed twice with 5 volumes of saline and made up to 4 times their original volume with 0.025 M $NaHCO_3$. This served to dilute as well as hemolyze the cells. Muscle enzyme preparations were made by extracting the tissue according to the method of Glick (3); 5 cc. of 0.025 M bicarbonate were used per gm. of tissue. The extract was then centrifuged and the supernatant used for the determination of enzyme activity. Brain extracts were made according to the method of Glick (3), that of Mendel and Rudney (4), or by homogenization of the tissue with 5-fold its weight of 0.025 M $NaHCO_3$ in a Waring blendor. 0.5 cc. of such supernatant fluids or suspensions was used for determination of enzyme activity.

The measurement of reaction velocity was taken as that volume of CO_2, in c.mm., produced in 30 minutes during the straight line portion of the time-action curve. Correction was made for non-enzymic hydrolysis of acetylcholine. As will be shown later, for rabbit serum a straight line relationship holds for the first 40 per cent hydrolysis of the substrate, and for periods usually longer than 30 minutes. In those instances in which direct proportionality between time and extent of action did not hold for as long an interval as 30 minutes, the CO_2 produced during the initial straight line portion of the reaction was taken as the measure of the reaction velocity and calculated for a 30 minute period. In the study of the hydrolysis of esters other than acetylcholine, the technique of measuring the activity was the same as that described above. The concentrations of these esters have been indicated at the appropriate places in the text or tables.

Rabbits, monkeys, and men were exposed to several concentrations of diisopropyl fluorophosphate for varying periods of time, as described in the text. In certain experiments, freshly prepared solutions of diisopropyl

fluorophosphate in saline were injected intravenously. Samples of blood, for determination of cholinesterase activity, were taken before and at various desired intervals after exposure to or injection of the fluorophosphate. For purposes of comparison, enzyme activities were expressed as per cent of the cholinesterase activity before exposure or before injection. Each animal thus served as its own control for the red blood cell and serum studies. Normal brain cholinesterase activities were determined on a series of untreated animals.

Samples of the inspired air, containing diisopropyl fluorophosphate, were withdrawn during the course of the experiments, and the concentration of diisopropyl fluorophosphate that the animals had been exposed to was determined. In those cases in which animals were sacrificed, they were killed by the intravenous injection of air and the brain was removed.

Results

Kinetics of Action of Cholinesterase on Acetylcholine in Presence of Diisopropyl Fluorophosphate—A considerable number of enzyme reactions are of zero order during the initial stage. This relationship has been shown to hold for cholinesterase (5, 6). In the present study, the amount of acetylcholine used should, upon complete hydrolysis, yield 1344 c.mm. of CO_2 at standard conditions. In Fig. 1 it may be seen that the action of rabbit serum cholinesterase for 130 minutes resulted in the production of 538 c.mm. of CO_2 or 40 per cent hydrolysis of the substrate. During this period, the time-action plot is a straight line. This relationship also held in the presence of diisopropyl fluorophosphate. The reaction was of zero order in the presence of diisopropyl fluorophosphate and indicated that the inhibitor was not producing a progressive inactivation of the enzyme. The hydrolysis proceeded as if less enzyme were present. Other types of cholinesterases also showed a zero order reaction during the first 40 per cent hydrolysis of 0.015 M acetylcholine. Among these were human muscle, human and monkey red blood cell, and human serum cholinesterases. On the other hand, with monkey serum cholinesterase there was a definite deviation from a zero order reaction during this portion of the hydrolysis. Thus in one case of monkey serum the amount of CO_2 liberated from 10 to 40 minutes after the start of the reaction was 128 c.mm., from 40 to 70 minutes 89 c.mm., from 70 to 100 minutes 86 c.mm., and from 100 to 130 minutes 60 c.mm. The presence of diisopropyl fluorophosphate tended to accentuate this progressive decrease in reaction velocity.

In Vitro Inhibition of Cholinesterase Activity by Diisopropyl Fluorophosphate and by Physostigmine—The inhibition of the activities of serum, red blood cell, muscle, and brain cholinesterases of the rabbit, monkey, and man was determined at various concentrations of diisopropyl fluorophos-

phate and physostigmine. In Table I are shown the cholinesterase activities of rabbit, monkey, and human sera at concentrations of fluorophosphate ranging from 10^{-3} to 10^{-10} M. The reaction velocities are expressed as per cent of the reaction velocities of the cholinesterases in the absence of the fluorophosphate. In order to obtain a general measure of the extent of inhibition of the different cholinesterases, the relative velocities were plotted against the negative logarithm of the molar concentration of diisopropyl fluorophosphate or physostigmine. The negative log molar con-
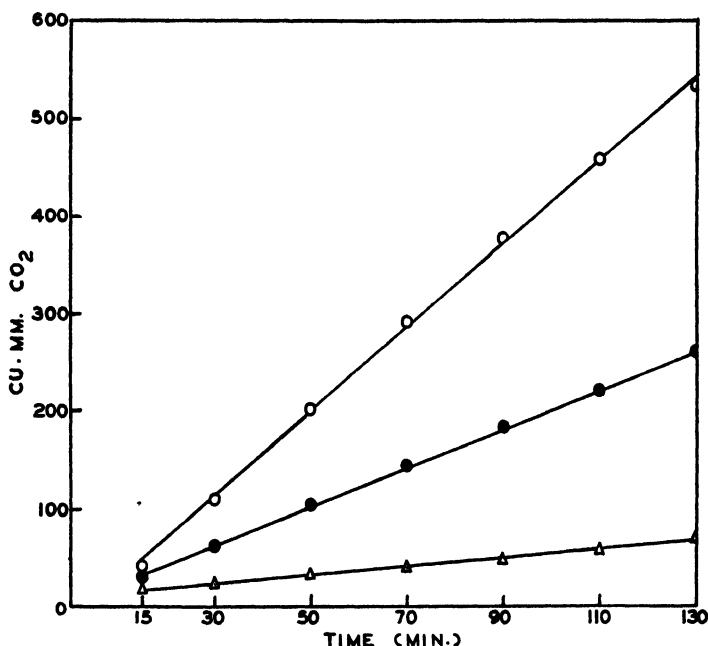
FIG. 1. Effect of diisopropyl fluorophosphate on the course of hydrolysis of acetylcholine by rabbit serum. ○ represents hydrolysis in absence of inhibitor, ● in presence of 1×10^{-4} M, and △ in presence of 5×10^{-4} M diisopropyl fluorophosphate.

centration at which 50 per cent inhibition occurred was termed the pC_I value. These values for various enzyme preparations are shown in Table II.

It may be seen that of the various serum cholinesterases studied that of the rabbit was least sensitive to inhibition by diisopropyl fluorophosphate. Thus a negative log molar concentration of 4.1 of diisopropyl fluorophosphate was necessary for 50 per cent inhibition of rabbit serum cholinesterase, whereas concentrations about 0.0001 as much (negative log molar values of 7.7 to 8.3) gave 50 per cent inhibition of monkey, human, and

TABLE I

In Vitro Inhibition of Rabbit, Monkey, and Human Serum Cholinesterase by Diisopropyl Fluorophosphate

Negative log molar concentration of diisopropyl fluorophosphate	Serum cholinesterase activity		
	Rabbit	Monkey	Man
	per cent of normal	*per cent of normal*	*per cent of normal*
3.0	7		8
3.3	10		
4.0	42	0	9
4.3	55		
4.6	67		
5.0	76	0	10
5.3	80		
5.5	82		
5.6			9
5.8	85		
6.0	85	3	
6.3	91		
7.0		5	9
7.3			29
7.6		51	49
7.8			59
8.0		75	
8.3			74
8.6		93	
9.0	96	95	
10.0			85

TABLE II

Sensitivity of Various Cholinesterases to Inhibition by Diisopropyl Fluorophosphate and Physostigmine

The values are expressed in terms of the negative log of the concentration of inhibitor required to produce a 50 per cent inhibition of cholinesterase activity (pC).

		Serum	Red blood cells	Muscle	Brain
Diisopropyl fluorophosphate	Rabbit	4.1	5.2		5.5
	Monkey (*Macacus rhesus*)	7.8	5.5		5.5
	Human	7.7	5.4	5.4	6.0
	Horse*	8.3			
	" (purified)	8.1			
Physostigmine	Rabbit	5.9			
	Human	6.7		6.1	

* Purified horse serum cholinesterase was obtained from Dr. Kunitz and Dr. Northrop.

horse serum cholinesterase activity. The various red cell cholinesterases showed approximately the same degree of sensitivity toward inhibition by diisopropyl fluorophosphate (pC_I values of 5.2 to 5.5), whereas human brain cholinesterase (pC_I = 6.0) was somewhat more sensitive than monkey or rabbit brain cholinesterase (pC_I = 5.5) to inhibition by diisopropyl fluorophosphate. Purified cholinesterase of the ·electric eel gave a pC_I value of 4.1.[4]

As will be seen later, when the *in vivo* results are discussed, it is of interest to compare the sensitivities of the various tissue cholinesterases from one species to inhibition by diisopropyl fluorophosphate. Rabbit brain and red blood cell cholinesterases showed a greater sensitivity than did serum cholinesterase; brain showed the greatest sensitivity. Thus, a 50 per cent inhibition of serum cholinesterase activity occurred at a negative log of the molar concentration of diisopropyl fluorophosphate of 4.1, whereas the same degree of red cell and brain cholinesterase inhibition occurred at values of 5.2 and 5.5 respectively. Monkey serum cholinesterase was much more sensitive to inhibition by diisopropyl fluorophosphate than red cell or brain cholinesterase. Human serum cholinesterase was much more sensitive to inhibition by diisopropyl fluorophosphate than human red cell or brain cholinesterase. This picture is similar to that found in the monkey and is in marked contrast to that observed in the rabbit.

Table II also shows the sensitivities of rabbit serum, human serum, and muscle cholinesterases to inhibition by physostigmine. It may be seen that rabbit serum cholinesterase was more sensitive to inhibition by physostigmine (pC_I = 5.9) than by diisopropyl fluorophosphate (pC_I = 4.1), whereas the reverse was true with human serum cholinesterase.

The possibility existed that the differences in sensitivity of different cholinesterases to inhibition by diisopropyl fluorophosphate were due to materials, other than the enzymes themselves, present in the preparations. Table II shows that a purified horse serum cholinesterase preparation had, within experimental error, the same pC_I value as horse serum itself. Table III illustrates one of several experiments which were carried out to show that sensitivity to inhibition by diisopropyl fluorophosphate was not dependent upon accompanying materials present in enzyme preparations. Human brain extract was heated to destroy its cholinesterase activity and added to human serum. The cholinesterase activity of the serum-brain mixture was then determined at various concentrations of diisopropyl fluorophosphate. The pC_I value for the mixture was 7.7, the same as that found for human serum cholinesterase itself (Table II).

In Vivo Inhibition of Cholinesterase Activity by Diisopropyl Fluorophosphate—In Table IV, *A* are presented the results of the effect of diisopropyl

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[4] This preparation was obtained from Dr. D. Nachmansohn.

fluorophosphate inhalation on the serum, red blood cell, and brain cholinesterase activity of rabbits. It may be seen that at the higher exposures symptoms were acute; one animal, Rabbit 354, died in the gas chamber and another, Rabbit 356, died 10 minutes after the beginning of exposure; the third, Rabbit 355, survived. The red cell cholinesterase activities in all of the animals after exposure were zero. Some cholinesterase activity was present in the serum of the two rabbits, Nos. 355 and 356. It may be recalled that the *in vitro* results indicated that rabbit red cell cholinesterase was more sensitive to inhibition by diisopropyl fluorophosphate than serum cholinesterase. This effect paralleled the *in vivo* results. At the next lower exposure, no symptoms except miosis were observed, although de-

TABLE III

Influence of Inactivated Brain Extract on Inhibition of Human Serum Cholinesterase by Diisopropyl Fluorophosphate

Composition of mixture	CO_2 liberated in 30 min.	Cholinesterase activity
	c.mm.	*per cent*
Serum	637	100
Inactivated brain extract	0	0
Serum + inactivated brain extract	651	102
" + " " " + diisopropyl fluoro- phosphate (10^{-7} M)	0	0
Serum + inactivated brain extract + diisopropyl fluoro- phosphate (5×10^{-8} M)	245	38
Serum + inactivated brain extract + diisopropyl fluoro- phosphate (2.5×10^{-8} M)	346	53
Serum + inactivated brain extract + diisopropyl fluoro- phosphate (10^{-8} M)	372	57

creases in the red cell serum and brain cholinesterase activities were obtained.

Because of the known breath-holding characteristics of the rabbit, the variation in respiratory intake, and other difficulties in determining the precise amount of diisopropyl fluorophosphate absorbed by the animal, the tissue cholinesterase activity was studied in rabbits which were injected intravenously with diisopropyl fluorophosphate solutions. The results are shown in Table IV, B. One rabbit, injected with 3.0 mg. per kilo of the fluorophosphate, died immediately; the serum, red cell, and brain cholinesterase activities were zero. Two rabbits were injected with 0.3 mg. per kilo of the fluorophosphate, a dose from which the animals usually recovered. Marked muscular tremors resulted, starting 3 to 5 minutes after the injection. Blood was taken for cholinesterase determinations 23

and 26 minutes after the injection. The animals were then sacrificed and the brains removed. The brain cholinesterase activities were 5 and 12 per cent of normal. The red cell cholinesterase activities were 0 and 7 per cent and the serum 5 and 15 per cent, respectively, of the preinjection

TABLE IV

Effect of Diisopropyl Fluorophosphate on Rabbit Plasma, Red Blood Cells, and Brain Cholinesterase Activity in Vivo

A. Inhalation

Rabbit No.	Concentration of diisopropyl fluorophosphate vapor	Duration of exposure	Blood sample time	Relative cholinesterase activity*			
				Plasma	Red cells	Brain	Remarks
	mg. per l.	*min.*	*min.*				
354	1.53	5	2	0	0		Died
355	1.53	5	5	20	0		Muscle tremors Recovered
356	1.53	5	8	11	0		Died in 5 min.
325	0.46	10	15	5	5	1	No symptoms
350	0.46	10	18	9	1	24	" "
351	0.46	10	21	13	10	2	" "
352	0.14	10	4	66	100		" , "
353	0.14	10	5	41	62		" "

B. Intravenous Injection

Rabbit No.	Dose	Blood sample time	Relative cholinesterase activity*			
			Plasma	Red cells	Brain	Remarks
	mg. per kg.	*min.*				
327	3.0	4	0	0	0	Died immediately
326	0.3	23	15	7	12	Muscle tremors
328	0.3	26	5	0	5	" "
491	0.1	26	54	41	59	No symptoms
492	0.1	25	37	29	57	" "
330	0.05	27	60	19	74	" "
331	0.05	43	51	29	73	" "

* These values are per cent of the preexposure values.

values. It may be noted that the red cell cholinesterase activities were lower than the serum activities.

At a dose of 0.1 mg. per kilo of diisopropyl fluorophosphate, the rabbit brain cholinesterase activities were 57 and 59 per cent of normal. There were no systemic symptoms at this dose. At a dose of 0.05 mg. per kilo of

diisopropyl fluorophosphate, the brain activities were 73 and 74 per cent of normal. These results indicated that cholinergic symptoms in the rabbit appear at an intravenous dose of between 0.1 and 0.3 mg. per kilo of the fluorophosphate. The brain cholinesterase activities, corresponding to the appearance of symptoms, were less than approximately 60 per cent of normal.

Table V, A shows the serum and red blood cell cholinesterase activities after exposure of *Macacus rhesus* monkeys to various concentrations of diisopropyl fluorophosphate vapor. It may be seen that the serum cholinesterase activity decreased markedly to from 0 to 5 per cent of the pre-exposure value even after exposure to very low concentrations of the fluorophosphate. The red cell cholinesterase activities decreased as a result of the exposure, but not as markedly as did the serum cholinesterase activities. The unusual sensitivity of monkey serum toward diisopropyl fluorophosphate *in vivo* paralleled that observed *in vitro*. As will be recalled, the pC_I value for monkey serum cholinesterase was 7.8. The finding that the red cell cholinesterase activity was not decreased as much as the serum cholinesterase after exposure parallels the differing sensitivities of these cholinesterases to inhibition by diisopropyl fluorophosphate *in vitro*. Of the four animals exposed in the chamber, only Monkey C showed symptoms; these were marked tremors, salivation, and difficulty in respiration. The occurrence of symptoms was accompanied by zero red cell cholinesterase activity after exposure. As may be seen in Table V, A, the return to normal of the serum cholinesterase was slow. Thus, 7 to 8 days after exposure the activity ranged from 39 to 55 per cent of normal.

Several monkeys were injected intravenously with diisopropyl fluorophosphate. The results are given in Table V, B. A dose of 0.3 mg. per kilo of the fluorophosphate was fatal in 10 minutes. The serum and brain cholinesterase activities were zero and the red cell activity but 2 per cent of normal. Essentially the same results were obtained at doses of 0.25 and 0.2 mg. per kilo, except that the survival period was longer. At a dose of 0.1 mg. per kilo the animal survived, although the serum and red cell cholinesterase activities were low.

These results indicate that in the rhesus monkey the occurrence of symptoms was associated with low brain cholinesterase activity. It may also be seen that a very low dose (0.02 mg. per kilo) decreased the serum activity to 2 per cent of normal, whereas it affected, to only a small extent, the brain cholinesterase activity. This finding parallels the *in vitro* difference in sensitivity between monkey serum and brain cholinesterase to inhibition by diisopropyl fluorophosphate. The marked *in vitro* sensitivity of monkey serum cholinesterase to low doses of the fluorophosphate is in contrast to that shown by the rabbit and, as will be shown presently, is strikingly similar to that in man.

One group of seven men was exposed to a concentration of 19 γ per liter of diisopropyl fluorophosphate vapor for 8 minutes, 42 seconds, a second

TABLE V

Effect of Diisopropyl Fluorophosphate on Monkey Plasma, Red Blood Cells, and Brain Cholinesterase Activity in Vivo

A. Inhalation

Monkey	Concentration of diisopropyl fluorophosphate vapor	Duration of exposure	Blood sample time	Relative cholinesterase activity*		Remarks
				Plasma	Red cells	
	mg. per l.	*min.*				
A	0.15	1.25	3 min.	5	68	No symptoms
			8 days	39	96	
C	0.15	1.25	4 min.	2	0	Muscle tremors, salivation, bronchial constriction
			9 days		27	Recovered
B	0.005	30	5 min.	1	63	No symptoms
			7 days	55	73	
	0.008†	30	11 min.	0	17‡	" "
D	0.008	15	3 "	1		" "
			7 days	46	6	
	0.198†	1.25	28 min.	1§	20‖	" "

B. Intravenous Injection

Monkey No.	Dose	Blood sample time	Relative cholinesterase activity¶			Remarks
			Plasma	Red cells	Brain	
	mg. per kg.					
4	0.3	10 min.	0	2	0	Died in 10 min.
6	0.25	27 "	0	1	0	" " 33 "
3	0.2	2 hrs.	0	0	0	Severe symptoms; sacrificed
1	0.1	24 min.	0	2		No symptoms
		5 days	86	38		
2	0.1	3.75 hrs.	0	10	0	Muscle tremors, diarrhea
5	0.02	60 min.	2	14	78	No symptoms

* All values given represent per cent of preexposure values.
† Second exposures of Monkeys B and D, 7 days after the first exposure.
‡ Equivalent to 12 per cent of the serum activity before the first exposure.
§ Equivalent to 0.4 per cent of the serum activity before the first exposure.
‖ The per cent activity of the red cells just before the second exposure.
¶ Per cent of preinjection value.

group of six men to a concentration of 27.1 γ per liter for 9 minutes, and two men to 28.8 γ per liter for 10 minutes, 40 seconds, and 27 γ per liter

for 6 minutes, 40 seconds, respectively. The symptoms were extremely mild. All of the men showed miosis and most of them complained of a slight feeling of tightness in the chest, lasting for several hours. The following symptoms were observed occasionally: increased nasal secretion, nausea, salivation.

Table VI shows the decreases in serum cholinesterase activity immediately after exposure and the rate of return of the activity to normal. It may be seen that, immediately after gassing, the serum cholinesterase activity decreased to about 1 to 5 per cent of the preexposure value. The

TABLE VI

Effect of Inhalation of Diisopropyl Fluorophosphate Vapor on Serum Cholinesterase Activity in Vivo in Man

Subject	Concentration of diisopropyl fluorophosphate vapor	Duration of exposure	Relative cholinesterase activity at various intervals after exposure*							
			5–30 min.	Days						
				1	2	3	4	8	10	15
	mg. per l.	min.								
J. H.	0.019	8.7	4	13			29	58		71
R. L.	0.019	8.7	3	7			28	53		77
W. B.	0.019	8.7	2	14			3	55		72
M. G.	0.019	8.7	3	12			31	50		69
J. P. M.	0.019	8.7	1	7			30	52		68
J. M.	0.019	8.7	3	17			22	57		82
T. M.	0.019	8.7	1	8			30	50		60
A. F.	0.027	6.7	3	13	23	16	29		50	62
H. H.	0.027	9.0	3†			29				
J. J. H.	0.027	9.0	5‡			26				
W. C.	0.027	10.7	1	5			26	49		69

* These values are per cent of the preexposure values.

† The red blood cell cholinesterase activity was 97 per cent of the preexposure value.

‡ The red blood cell cholinesterase activity was 87 per cent of the preexposure value.

rate of return to normal was very slow. On the average, the activity returned to about 30 per cent in 4 days, to about 50 per cent in 8 days, and to about 70 per cent in 15 days. The red cell cholinesterase activities of several of these men were determined immediately after exposure and were found to be only slightly decreased below preexposure values. These results show, therefore, a correlation with the in vitro sensitivities of human serum and red cell activities.

Rate of Restoration of Rabbit Plasma, Red Blood Cell, and Brain Cholinesterase Activities after Poisoning with Diisopropyl Fluorophosphate—The slow rate of regeneration of serum cholinesterase activities, demonstrated

above in man and monkey, raised the question as to the rate of regeneration of brain cholinesterase activity. The average brain cholinesterase activity was first determined in a series of normal rabbits. Each of a group of about fifty rabbits was injected with 0.3 mg. of diisopropyl fluorophosphate per kilo and blood samples were taken before injection for determination of normal plasma and red cell cholinesterase. At this dose, the rabbits developed tremors within about 15 minutes after injection and continued to have these tremors throughout the day. About 10 per cent of the animals died. The surviving animals were free of symptoms the day following injection. At suitable intervals after injection, blood was
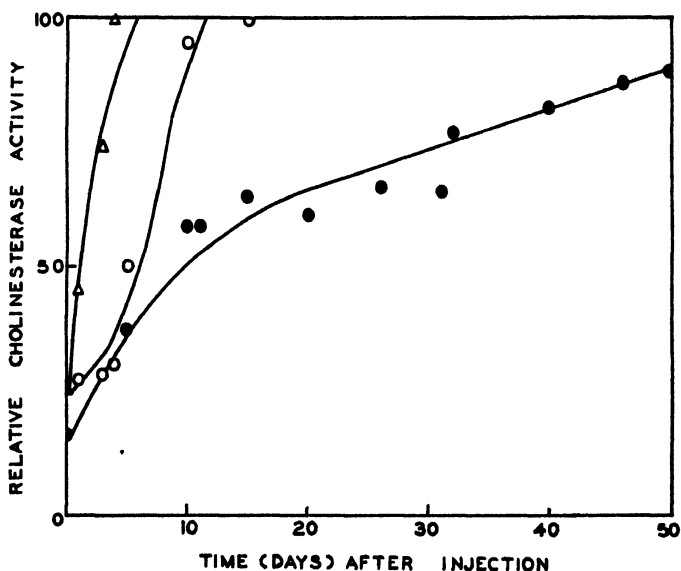
FIG. 2. Regeneration of cholinesterase activity of serum (Δ), red blood cells (O), and brain (●) in the rabbit, after intravenous injection of 0.3 mg. of diisopropyl fluorophosphate per kilo.

taken for determination of plasma and red cell cholinesterase activities. At these or other times after injection, one to six rabbits were selected for sacrifice, and brains were removed within 5 minutes after death and the brain cholinesterase activities determined.

Fig. 2 shows that the recovery of plasma cholinesterase activity to normal values occurred in about 5 days. The red cell cholinesterase activity returned to normal somewhat more slowly. It was about 50 per cent of normal in about 5 days and attained the normal, preinjection level in 10 days. The rate of recovery of brain cholinesterase activity was exceedingly

slow.   10 days after injection the brain cholinesterase activity was about
50 to 60 per cent of normal.   20 to 30 days after injection, it was about 60
to 70 per cent of normal.   50 days after injection, the brain cholinesterase
activity had returned to 90 per cent of normal.

*Attempts at Reversal of Diisopropyl Fluorophosphate Inhibition of Cholin-
esterase*—It has been shown that the inhibitions of phosphatase by amino
acids (7), of pepsin by proteolytic digestion products (8), and of cholin-
esterase by physostigmine (9, 10) may be reversed by subjecting the en-
zyme-inhibitor mixture to dialysis or dilution.   In the present study, the
serum and brain extracts of rabbits injected with 0.3 mg. per kilo of di-
isopropyl fluorophosphate were dialyzed against several changes of saline
for about 24 hours.   In a typical experiment, the cholinesterase activity
of the serum was found to be 14 per cent of normal before, and 12 per cent
after dialysis.   The brain cholinesterase activity was 12 per cent of normal
before, and 14 per cent of normal after dialysis.   Rabbit plasma was also
treated *in vitro* with diisopropyl fluorophosphate and then dialyzed for
24 hours; the cholinesterase activity was 28 per cent of normal before, and
24 per cent of normal after dialysis.   These results show that under these
conditions dialysis did not result in any reversal of the fluorophosphate
inhibition of cholinesterase activity.   The *in vitro* results are in agreement
with those of Mackworth.[3]

The following experiments show that dilution of a mixture of cholin-
esterase and fluorophosphate did not result in any reversal of inhibition.
The cholinesterase activity of 0.5 cc. of a normal human plasma was 843
c.mm. of CO_2 liberated in 30 minutes; the activity of 0.05 cc. of this plasma
was 93 c.mm. liberated in 30 minutes, or 11 per cent.   This plasma was
treated with an appropriate concentration of diisopropyl fluorophosphate
so that 0.5 cc. had an activity of 405 c.mm. of CO_2 liberated in 30 minutes;
0.05 cc. of this treated plasma showed an activity of 42 c.mm., or 10 per
cent.   Thus dilution did not result in any relative increase in activity.
This may be contrasted with the result in an experiment on human plasma
treated with physostigmine; 0.5 cc. of this plasma showed an activity of
55 c.mm. of CO_2 liberated in 30 minutes, whereas 0.05 cc. of this plasma
showed an activity of 30 c.mm., or 55 per cent.   This relative increase in
cholinesterase activity on dilution of a physostigmine-cholinesterase mix-
ture is, of course, in agreement with a previous report (10).

DISCUSSION

The present study has concerned itself with a new anticholinesterase
compound, diisopropyl fluorophosphate.   Of particular interest has been
the finding that different tissue cholinesterases show differing degrees of

sensitivity to inhibition by diisopropyl fluorophosphate. It has been shown that there is a parallelism between these *in vitro* sensitivities and the extents to which the various tissue cholinesterases examined were inhibited *in vivo* after exposure to or injection with diisopropyl fluorophosphate.

In view of the difference among the cholinesterases of different tissues to inhibition by diisopropyl fluorophosphate, it would be unjustified to draw any conclusions from our data concerning the sensitivity to inhibition of cholinesterases at autonomic effector organs, ganglia, or myoneural junctions. According to the concept of chemical transmission of nervous impulses, the extent of cholinesterase inhibition at these sites should be correlated with the appearance of various cholinergic symptoms. In general, in the monkey or rabbits, such cholinergic symptoms as muscular tremors, salivation, and diarrhea were associated with low red cell and brain cholinesterase activity, and death was associated with zero brain cholinesterase activity. However, this association is to be regarded as fortuitous. Conversely, it should be emphasized that depression of serum cholinesterase activity does not necessarily indicate the appearance of cholinergic symptoms; in monkey and man, for example, the serum cholinesterase activity may be reduced to extremely low levels without the manifestations of such symptoms.

In confirmation and extension of previous work,[3] the present study has shown that the inhibition of cholinesterase by diisopropyl fluorophosphate is, in contrast to the inhibition by physostigmine, not reversed by dilution or dialysis of the enzyme-inhibitor mixture. If, as is generally assumed (11), an inactive enzyme-inhibitor complex is formed, then such a complex is not readily dissociated. The possibility exists, of course, that the active enzyme might be regenerated by combining the inhibitor with some other substance to form a less dissociated complex than the enzyme-inhibitor complex.

The persistence of low serum, red cell, and brain cholinesterase activity in the rabbit for periods of 5, 10, and 60 days, respectively, and of low serum cholinesterase activities in the monkey and man for periods of at least 1 to 2 weeks, offers evidence in support of the irreversibility of inactivation *in vivo*. Hall and Ettinger (12) found that, after injections of physostigmine in the dog, the serum cholinesterase activity dropped to 10 to 25 per cent of normal in about a half hour and returned to normal in 2 hours. This prompt restoration of normal activity may well be expected in the case of a readily reversible inhibitor-enzyme complex. On the other hand, the long periods of time necessary for the restoration of normal cholinesterase activity, after exposure to diisopropyl fluorophosphate vapor or injections with diisopropyl fluorophosphate solutions in the instances

mentioned, are of the same order of magnitude as those necessary for the regeneration of protein (13) and would seem to indicate a synthesis of enzyme protein.

SUMMARY

1. The inhibition of serum, red blood cell, brain, and muscle cholinesterase activity of the rabbit, monkey, and man by a wide range of diisopropyl fluorophosphate concentrations was studied. For purposes of comparison, the inhibition by physostigmine of rabbit and human serum and muscle cholinesterase activity was also investigated. The degree of inhibition was quantitatively evaluated as the negative log molar concentration of diisopropyl fluorophosphate necessary to produce a 50 per cent decrease in the original cholinesterase activity. For convenience, this is described as the pC_I value.

2. The effect of exposure to, or injection with, diisopropyl fluorophosphate on the cholinesterase activity of the serum, red blood cell, and brain was studied in the rabbit and monkey. The effect of exposure to low concentrations of diisopropyl fluorophosphate on human serum cholinesterase activity was also investigated. It was found that cholinesterases, such as those of human and monkey serum, which were very sensitive *in vitro*, showed marked *in vivo* decreases without the accompanying development of cholinergic symptoms.

3. The rate of restoration of serum cholinesterase activity in monkey and man was very slow. In the rabbit, 5, 10, and about 60 days were necessary for the restoration to normal of serum, red cell, and brain cholinesterase activities, respectively.

4. Dialysis or dilution of cholinesterase-diisopropyl fluorophosphate mixtures or of tissue cholinesterases from animals poisoned with diisopropyl fluorophosphate did not result in any increase of the cholinesterase activity. These experiments indicated that the inhibition of cholinesterase by diisopropyl fluorophosphate was not readily reversible.

5. The results on diisopropyl fluorophosphate inhibition of cholinesterase activity have been discussed with regard to the questions of cholinesterase irreversibility and the appearance of cholinergic symptoms.

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SOME CULTURE STUDIES ON LACTOBACILLUS ARABINOSUS AND LACTOBACILLUS CASEI*

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Probably one of the most important factors in successful microbiological analysis is the method of culture of the bacterial organisms employed in each test. It is only when the bacterial cells are in optimum nutrition that they can be expected to respond properly as test organisms. It is not an uncommon experience that cultures of test organisms do not always give reliable standard curves. Furthermore, it is frequently observed that on prolonged culturing the bacteria may exhibit a gradual decrease in total acid production and in linearity of response to the nutrient under test. Such occurrences limit the useful range of the standard curves and may cause doubt as to the validity of data resulting from the analysis of test substances. Such comment has also been made by Krehl, Strong, and Elvehjem (1).

In this paper studies are reported on the effect of the method of culture upon the response of *Lactobacillus arabinosus*[1] and *Lactobacillus casei*.[2]

EXPERIMENTAL

Culture Media Employed. Preparation of Liver Extract—At first a liver concentrate paste was used as a source of liver nutrients. However, an aqueous extract of fresh liver was later used and considered to be a better source of nutrients. It is made as follows: 1 pound of ground fresh liver is suspended in 2 liters of water, heated 60 minutes on a steam bath, and then filtered through cheese-cloth. After neutralization to pH 7.0, it is heated again for 15 minutes, filtered through coarse filter paper, and stored under toluene in a dark bottle in the refrigerator.

The agar and broth media used in these culture studies have the following composition per 100 ml.: *yeast-glucose agar*, 1 gm. of glucose, 1 gm. of yeast extract (Difco), and 1.5 gm. of agar; *liver-tryptone agar*, 1 gm. of tryptone (Difco), 1 gm. of glucose, 0.2 gm. of K_2HPO_4, 0.3 gm. of $CaCO_3$,

[1] American Type Culture Collection, No. 8014.

[2] American Type Culture Collection, No. 7469.

1.5 gm. of agar, 10 ml. of liver extract, and 0.5 ml. each of Salts A and B;[3] *liver-tryptone broth*, 1 gm. of tryptone (Difco), 0.5 gm. of K_2HPO_4, 0.2 gm. of glucose, 0.2 gm. of yeast extract (Difco), and 10 ml. of liver extract.

Effect of Culture Method upon Response of Lactobacillus arabinosus—It is evident that there are a number of variations in the manner in which stock cultures of *L. arabinosus* are carried. In the original paper of Snell and Wright (2) on niacin, stock cultures of *L. arabinosus* are carried in yeast extract-glucose agar and transferred at monthly intervals, being incubated at 30° for 24 to 48 hours. Inoculum for the assay tubes is prepared by a transfer from the stock culture to a tube of the basal medium containing 0.1 γ of niacin per ml. of solution, and is then incubated at 30° for 18 to 36 hours before use. Krehl, Strong, and Elvehjem (1) transfer the stock culture weekly in buffered yeast extract-glucose agar with incubation at 37°. Kent-Jones and Meicklejohn (3) report results obtained from stock cultures transferred monthly in solid glucose agar at 37°.

In this laboratory it was found that cultures transferred every 3 or 4 weeks in yeast extract-glucose agar with incubation at 37° gradually decreased in linearity of response to the added niacin. A new culture of *Lactobacillus arabinosus* obtained from the American Type Culture Collection gave a linear response up to 0.3 γ of niacin per tube. The acid production was not measured at a higher level of the vitamin at this time. After about 4 months the linearity had fallen to about 0.2 γ of niacin. Monthly transfer through liver-tryptone agar was also tried but this did not seem to improve the activity of *Lactobacillus arabinosus*. After 10 months this culture gave a linear response only up to about 0.15 γ of niacin. This decline in linearity limited, of course, the useful range of the standard curve and, at the same time, the organism may possibly give an unknown response to substances in the test solution, owing to its altered viability.

At the end of this period it was decided to attempt a "rejuvenation" of the microorganism. That is, it was decided to determine whether frequent transfer of the culture through nutritious media would result in a return of the original activity and linearity of response of the culture to the added vitamin. A series of transfers through skim milk and subsequent transfers through nutrient-rich liver-tryptone broth for several times and then weekly transfer from liver-tryptone agar to broth to agar again within 3 weeks resulted in marked improvement of response. Linearity had increased to 0.2 γ per tube. After this time the temperature of incubation was also changed to 30°, since this temperature is considered to be the optimum temperature for *Lactobacillus arabinosus* (4).

[3] The inorganic salts are as recommended by Snell and Wright (2). Solution A contains 25 gm. of potassium monohydrogen phosphate and 25 gm. of potassium dihydrogen phosphate in 250 ml. of water. Solution B contains 10 gm. of magnesium sulfate heptahydrate, 0.5 gm. of sodium chloride, 0.5 gm. of ferrous sulfate heptahydrate, and 0.5 gm. of manganese sulfate tetrahydrate dissolved in 250 ml. of water.

Further skim milk and liver-tryptone broth transfers increased the linearity within another 2 weeks to 0.25 γ of niacin. After 5 months (with weekly transfer through liver-tryptone broth to liver-tryptone agar for stock transfer, and from agar to broth to basal medium plus niacin
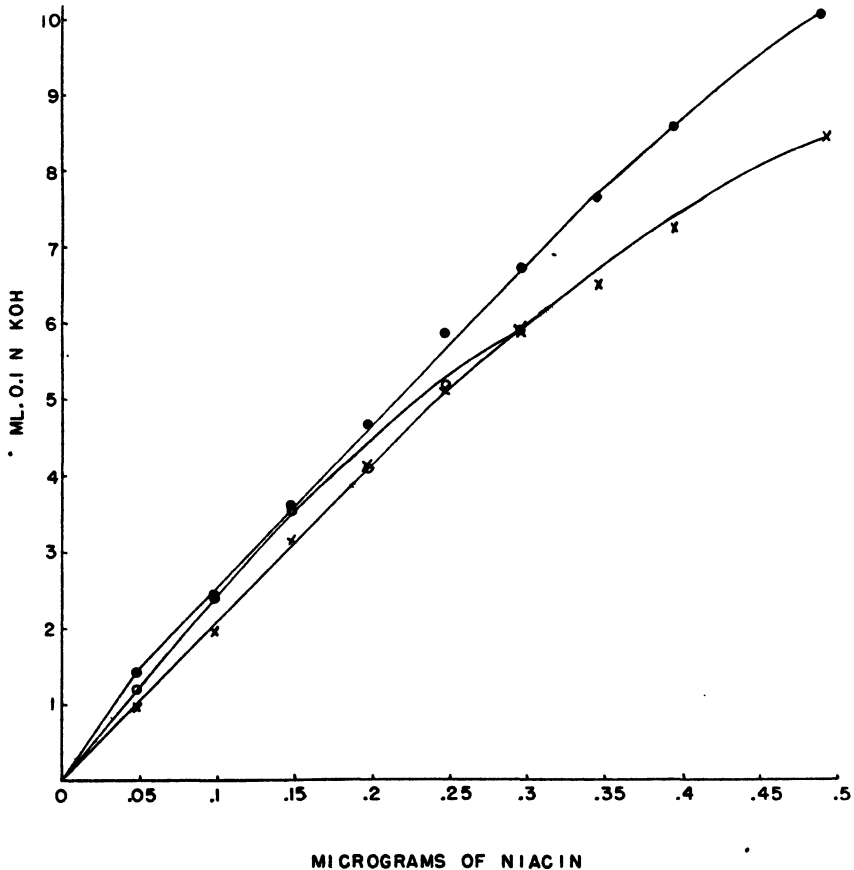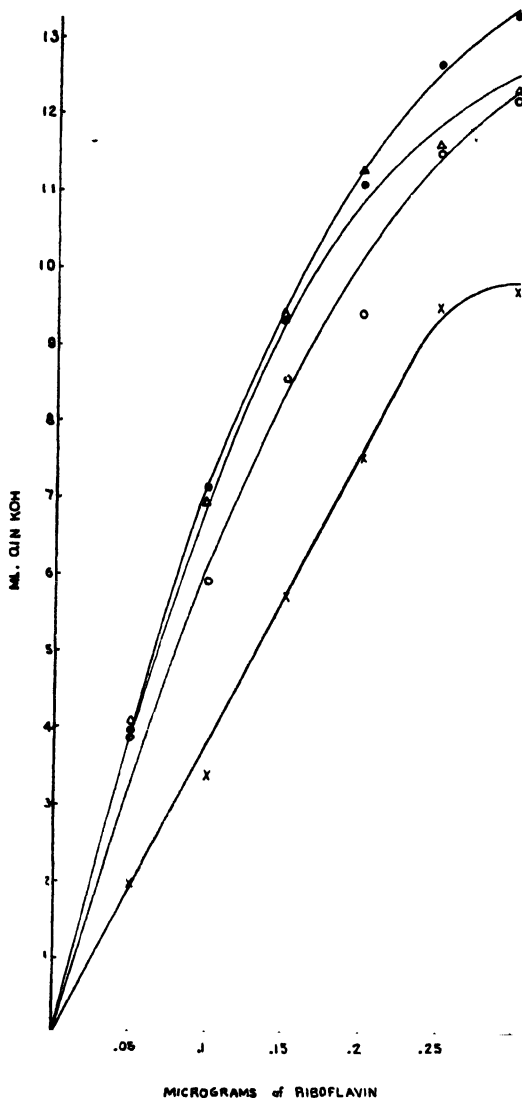

Fig. 1. Curves showing the effect of culture method upon the acid production of *Lactobacillus arabinosus* in response to added niacin. × (August 3, 1943) original culture carried in yeast-glucose agar at 37°; ○ (June 13, 1944) monthly transfer in liver-tryptone agar at 37° just prior to weekly transfer procedure; ● (December 11, 1944) 5 months after weekly transfer at 30°.

for the preparation of the inoculum) linearity to nearly 0.5 γ was attained. For the effect of various culture methods upon the response of *Lactobacillus arabinosus* to niacin, see Fig. 1.

Although this procedure of weekly transfer was more time-consuming than that of monthly transfer, the effort seemed worth while, since a

FIG. 2. Curves showing the effect of culture method upon the acid production of *Lactobacillus casei* in response to added riboflavin. X (August 3, 1943) original culture carried in yeast-glucose agar at 37°; O (May 7, 1944) monthly transfer in liver-tryptone agar at 37°; △ (October 10, 1944) 3 months after weekly transfer through liver-tryptone broth to liver-tryptone agar at 37°; ● (December 11, 1944) 5 months after weekly transfer at 37°.

standard curve of much greater reliability was obtained.   Not only was the useful range of the standard curve increased but also, since the bacterial

culture was in a state of better nutrition and of greater viability, it would not seem so likely to be affected by unknown substances in the test solution.

Effect of Culture Method upon Response of Lactobacillus casei—*L. casei*, the microorganism used in these studies for assaying riboflavin, was also affected by the culture method, as was *L. arabinosus*. *L. casei*, however, seems to be subject to a great many variations, for the reproducibility of standard curves from one assay date to another in no way parallels that of the standard curves obtained by the response of *L. arabinosus* to niacin.

The first curve obtained with the new culture of *Lactobacillus casei* was linear to 0.25 γ of riboflavin per assay tube. After that time the response of the organism to riboflavin resulted in a more rounded curve. The change to liver-tryptone agar from yeast-glucose agar with monthly transfer resulted in a definite increase in acid production with a much steeper curve. An attempted "rejuvenation" similar to that employed for *L. arabinosus* but with continued incubation at 37° also increased the acid production over and above that of the culture transferred through liver-tryptone agar every 3 or 4 weeks at 37°. However, it did not give as significant an improvement as did the change of agar from yeast-glucose agar. The yeast-glucose agar seems deficient in nutrients necessary for the maintenance of an active culture of *L. casei*. Broth transfer and frequency of transfer may also be of some importance. The effect of culture method upon the response of *L. casei* to riboflavin is shown in Fig. 2. The curves shown are based on values corrected with the blank titrations to zero in order to make them strictly comparable.

DISCUSSION

While these culture studies on *Lactobacillus arabinosus* and *L. casei* are by no means complete, they do emphasize the fact that the method of culture of the test organism is a very important factor in the response of the bacteria to the nutrients assayed. Many papers have been reported on the stimulatory effects of the addition of certain ingredients to the usual basal medium employed for a microbiological test, such as the effect of rice polishings concentrate on the response of *L. casei* reported by Clarke et al. (5, 6). Other stimulatory effects on *L. casei* by certain substances have been reported by Wegner et al. (7). The influence of buffer and glucose on the acid production of *L. casei* has been reported by Stokes and Martin (8). In the paper by Krehl, Strong, and Elvehjem (1) evidence has been presented to show that increased acid production and increased linearity of response to niacin were obtained with *L. arabinosus* when the basal medium of Snell and Wright (2) was modified and a different procedure in growing the inoculum was used.

In the studies on *Lactobacillus arabinosus* here reported the modified basal

medium of Krehl, Strong, and Elvehjem (a commercial casein hydrolysate was used rather than a laboratory preparation) has been used throughout for assay purposes. The culture method has proved to be one of the determining factors in the response of the microorganism to niacin. In the case of *Lactobacillus casei* there was a very marked increase in acid production by a change of agar medium. The more complex agar medium evidently supplied growth essentials or stimulants to acid production that were not present in the yeast-glucose agar or were present only in small amounts.

Further studies on culture methods for these organisms seem to be worthy of investigation, for it appears likely that culture method has an important rôle in the production of successful microbiological assays.

SUMMARY

Data have been presented which show that the method of culture affects the response of the microorganisms *Lactobacillus arabinosus* and *Lactobacillus casei* to niacin and riboflavin, respectively. Degraded cultures of these organisms can be "rejuvenated" by a change in transfer medium, greater frequency of transfer, and by incubation of the culture at the optimum temperature.

In the case of *Lactobacillus arabinosus*, a change of incubation temperature from 37° to 30° and weekly transfer of the stock culture from liver-tryptone agar to liver-tryptone broth to agar again after 5 months resulted in a change of linearity of response to added niacin from a maximum of 0.15 γ to about 0.4 γ of niacin. A change in agar medium from yeast-glucose to liver-tryptone agar without increased frequency of transfer of *Lactobacillus casei* with incubation at 37° resulted in a definite increase in acid production. Frequent transfer through broth to agar also effected some increase in acid production of *Lactobacillus casei*.

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CONCENTRATION OF THE RABBIT PAPILLOMA VIRUS WITH THE SHARPLES SUPERCENTRIFUGE*

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The purification of viruses has been accomplished thus far chiefly by the use of the vacuum type air-driven ultracentrifuge. The instrument, though of low volume capacity, has served effectively to provide small quantities of purified materials. There are occasions, however, when large scale work is desirable and necessary, particularly with respect to the initial concentration of relatively small quantities of virus from large volumes of crude tissue extract. An instrument long available and of much potential value because of its greater capacity is the laboratory model Sharples supercentrifuge. A number of workers (1–5) have demonstrated that viruses can be concentrated by means of this instrument, using either the standard equipment or various modifications of it. Stanley (4) was able to sediment 60 per cent of the tobacco mosaic virus out of large volumes of juice from infected tobacco plants. Subsequently the Sharples supercentrifuge was used for the sedimentation of influenza virus (6) and the preparation of influenza virus vaccines (7, 8). In recent work in this laboratory, relatively large quantities of purified papilloma virus were required for measurement of the density of the virus in solution (9). Accordingly, studies were made on purification of this virus with procedures involving use of the Sharples instrument. The results obtained are described in the present paper.

Materials and Methods

The Sharples supercentrifuge used was the air-driven laboratory model, modified as previously described in detail (7). The instrument was equipped with a standard *batch bowl* which had been converted into a continuous flow bowl of residual volume (50 ml.) smaller than that of the standard continuous flow bowl (160 ml.). The advantages of the modified bowl are (1) a thinner layer of fluid through which the virus must sediment

during centrifugation, and which thus permits a much more efficient concentrating action at higher rates of flow (7), (2) a small volume of concentrate for further processing, and (3) retention of the concentrate within the bowl at the end of the run. The instrument was mounted under ultraviolet lights (7) to prevent heavy contamination of the concentrate with viable bacteria.

The papillomas were naturally occurring growths obtained from cottontail rabbits trapped in Kansas. The warts were removed from the animals in the field, covered with a mixture of equal parts of glycerol and 0.9 per cent NaCl solution in 8 ounce bottles, and shipped immediately to the laboratory, where they were stored at 2–8°. The growths used in the present experiments were collected over a period of about 14 months and had been in glycerol-saline solution from 4 to 5 years. The number of bottles

TABLE I

Yield of Papilloma Virus Obtained by Preliminary Concentration from Crude Wart Extracts with Sharples Supercentrifuge and One, Two, or Three Cycles of Low and High Speed Spinning in Vacuum Ultracentrifuge

Batch No.	No. of bottles	Date interval bottles received	Weight of papillomas extracted	No. of cycles in vacuum ultra-centrifuge	Yield of virus	
					Total	Per gm. papillomas
			gm.		mg.	mg.
85-86	2	Dec. 28, 1939–Jan. 12, 1940	152.0	1	50.4	0.33
87-89	10	Jan. 17–Mar. 21, 1940		1	94.1	0.133
	7	Dec. 1, 1940–Jan. 10, 1941	708.0	2	83.5	0.118
				3	76.9	0.108
90	6	Jan. 10–Feb. 10, 1941	287.0	2	59.2	0.206

of warts and the dates they were received at the laboratory are given in Table I.

Use of the Sharples instrument for concentration of the papilloma virus is illustrated in the procedures of a typical experiment. 150 gm. of warts were freed of hair and extraneous tissue and broken up in about 100 ml. of 0.9 per cent NaCl solution in the Waring blendor. The pulp was made up to a volume of 2 liters (a 7.5 per cent tissue suspension) and set aside at 2–8° to extract overnight. The suspension was then mixed with 50 gm. of No. 512 Celite and filtered through a thin mat (2 to 3 mm.) of No. 503 Celite. The resulting, slightly turbid filtrate was passed into the Sharples supercentrifuge through a No. 24 gage hypodermic needle (7) at a constant rate of 500 ml. per hour. A centrifugal field of 62,000 g was maintained at the inside bowl periphery (50,000 R.P.M.). The extract was followed with

500 ml. of a solution containing 0.13 M NaCl and 0.05 M phosphate buffer, pH 6.5, to displace the final 50 ml. of extract and to wash the sedimented virus. As the speed of the bowl slowed, the lower inlet boss was stoppered (7). Removed from the machine, the bowl containing the concentrated virus in a volume of 50 ml. was set aside in the cold room overnight. After thorough shaking of the bowl the next morning, the 50 ml. of virus suspension were washed into a 100 ml. centrifuge tube with 20 ml. of the buffer-saline solution. This was spun in an angle centrifuge for 15 minutes at 3000 g and the supernatant fluid decanted. The soft, insoluble sediment was washed two times with 25 ml. portions of buffer-saline solution, and the wash fluids were added to the initial concentrate.

The virus recovered from an initial volume of 2 liters of crude extract was now in a volume of 120 ml. This was subjected to a single cycle of centrifugation in the vacuum type ultracentrifuge (5000 g for 5 minutes;
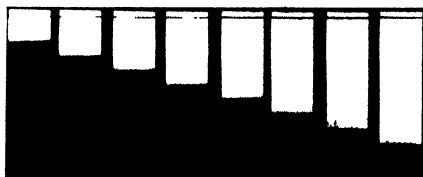
FIG. 1. Sedimentation diagram of papilloma virus concentrated from crude filtered extract with the Sharples supercentrifuge and purified by three cycles of low and high speed spinning in the vacuum ultracentrifuge. The virus concentration was 3 mg. per ml. The mean ultracentrifugal field was 17,000 times gravity and the interval between exposures 2.5 minutes. The sedimentation constant was $S_{20}° = 292$ Svedberg units.

50,000 g for 1 hour), after which the pellets were taken up in 6.5 ml. of the buffer-saline solution. Large aggregates were eliminated by low speed centrifugation in the vacuum ultracentrifuge at 5000 g for 5 minutes.

Results

The results of experiments with three different batches of warts are shown in Table I. The experiment described above involved Batch 85-86; 50.4 mg. of virus were obtained, a yield of 0.33 mg. of virus from 1 gm. of warts. Infectivity measurements[1] by the incubation period method (10) gave a 50 per cent point infectious unit of $10^{-8.6}$ gm. of virus, a value indicating a close similarity of the biological activity of this preparation with purified papilloma virus previously studied (10). In the experiment with Batch 87-89, the initial sedimentation in the Sharples instrument was

[1] These measurements were generously made by Dr. Alexander Hollaender and Dr. W. Ray Bryan, National Cancer Institute.

followed by three cycles of alternate low and high speed spinning in the vacuum ultracentrifuge. A considerable loss of material was experienced with each additional cycle, but the proportion of the loss consisting of actual virus was not estimated. A sedimentation diagram of Batch 87-89 concentrated in the Sharples supercentrifuge and sedimented three times in the vacuum ultracentrifuge is given in Fig. 1. The diagram indicates a high degree of homogeneity of the virus concentrate.

In previous experiments (11) on isolation of the papilloma virus with use of the vacuum ultracentrifuge throughout the process, approximately 1000 gm. of papillomas yielded 200 mg. of virus. This quantity of purified virus was collected in a series of twelve experiments in which the respective yields were extremely variable. In the present experiments 1147 gm. of papillomas yielded 186.5 mg. of purified virus, a result which compares favorably with the earlier findings. As noted above, the growths used here had been stored in 50 per cent glycerol-saline solution at 5–8° for a period of 4 to 5 years, and this apparently did not materially influence either the physical characters (9) or the biological activity (10) of the virus recovered.

<center>DISCUSSION</center>

The present work demonstrates the usefulness of the Sharples supercentrifuge in the preliminary concentration of the papilloma virus from tissue extracts of volumes inconveniently large for routine purification work with the vacuum ultracentrifuge. While the proportion of the virus recovered from the extracts was not determined, the yields were satisfactorily comparable with those obtained by the use of the vacuum ultracentrifuge alone. It is noteworthy that a filter paper lining (5) for the bowl was not employed; instead, the papilloma virus was deposited directly on the wall of the spinning bowl, as in the instance of the influenza virus (7) in previous work. Quantitative recovery of the virus from the bowl wall was greatly facilitated by stoppering the bowl before rotation had ceased.

The recent critical evaluation by Markham (5) of the theory of sedimentation in relation to the technique of "differential centrifugation" emphasizes properly certain practical limitations of the ultracentrifugal procedures generally utilized for the isolation of viruses. The ultracentrifugal process, as usually applied with the vacuum ultracentrifuge, is essentially one of washing away in the supernatant fluids of the high speed runs unsedimentable materials of particle size much smaller than that of the viruses, and removal by relatively very low speed centrifugation of gross tissue particles and material aggregated in the preceding high speed run. The centrifugal field differential employed is far too great to

permit appreciable separation of discrete components with sedimentation constants differing, for instance, by as much as $S = 70$ (12) and $S = 250$ Svedberg units (13). Even when the differences in sedimentation constants of the materials are markedly greater (14), considerable difficulty was encountered. The centrifugal field developed by the Sharples supercentrifuge was sufficient to sediment effectively the papilloma virus, despite convection associated with a continuous and relatively rapid flow of extract through the bowl. With the virus, however, there was sedimented much of the finely divided melanin pigment and other dispersed particles not removed from the extract by preliminary filtration with Celite. Markham (5) has indicated the lack of advantage of repeated centrifugation in the Sharples instrument in the presence of convection such as that occurring in the continuous flow bowl. In the vacuum ultracentrifuge, however, convection is a much smaller factor, and extraneous particles aggregated by packing in the pellets of the high speed runs can be eliminated by low speed spinning.

SUMMARY

The rabbit papilloma virus has been purified in satisfactory yields by preliminary sedimentation with the Sharples supercentrifuge from large volumes of extracts of cottontail rabbit warts, followed by repeated high and low speed spinning in the vacuum type air-driven ultracentrifuge. The combined procedures described provide access to large quantities of the virus in preparations of relatively high homogeneity, with very considerable savings in time and cost.

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THE DENSITY AND SIZE OF THE RABBIT PAPILLOMA VIRUS*

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A direct method of measuring the density of virus particles in suspension consists of sedimentation velocity studies of the virus in media of various densities (1–4). Unfortunately, this method is unsatisfactory when the density of the suspending medium is varied with materials such as NaCl, sucrose, urea, or glycerol because of alteration in the density of the particle under study. As a consequence, there occurs a breakdown of the linear relationship which should exist between the sedimentation rate of the virus and the density of the medium, and the resulting values of particle density are too high. This effect has been attributed to the osmotic pressure of the denser media (4).

A means for lessening the possible osmotic influence is by use of bovine serum albumin, which is of relatively high molecular weight, for varying the density of the suspending medium. Use of this material has given satisfactory results in studies on the density of the three types of influenza virus (5, 6) and, in more recent experiments, has been employed in a study of the density of the rabbit papilloma virus in aqueous suspension. The results thus obtained are given in the present report. For purposes of computation it was desirable first to determine the relation of sedimentation velocity to virus concentration, and the findings in this preparatory work are likewise described in the present paper.

Material and Methods

The papilloma virus was procured from extracts of cottontail rabbit warts by centrifugal concentration and purification. Some of the virus was purified in the usual way (7) by means of the air-driven quantity ultracentrifuge; most of it, however, was first concentrated in the Sharples

centrifuge and purified finally by repeated sedimentation in the quantity ultracentrifuge. The procedures involving use of the Sharples centrifuge and the product thus obtained have been described in a separate report (8).

Several different batches of concentrates were used separately for the present studies. The purified virus was suspended in concentrations of 6.3 to 9.6 mg. of virus per ml. in a solution of 0.13 M NaCl and 0.05 M phosphate buffer of pH 6.5. Estimation of virus content was made from Kjeldahl nitrogen determinations with a conversion factor (9) of 6.66. The character of the concentrates may be judged from the single sharp boundaries of the sedimentation velocity diagrams described elsewhere (8).
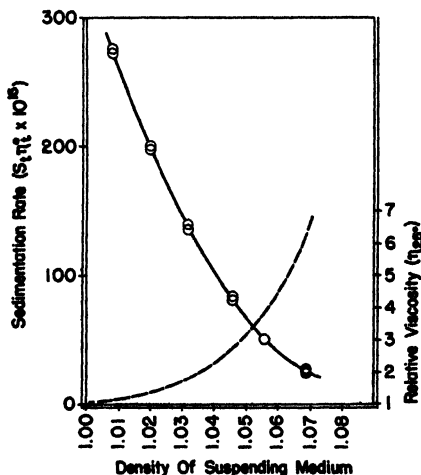
FIG. 1. Sedimentation of the rabbit papilloma virus in aqueous solutions of bovine serum albumin of various densities. The ordinate of the continuous line is the product of observed virus sedimentation rate and the viscosity of water at the rotor temperature and that of the broken line is the relative viscosity of the suspending media at 25°.

The bovine serum albumin was a crystalline fraction which Dr. Hans Neurath obtained from the Armour Laboratories, Chicago, Illinois, through the courtesy of Dr. E. J. Cohn and Dr. H. B. Vickery. The albumin was dissolved in approximately 25 per cent concentration in a solution containing 0.13 M NaCl and 0.05 M phosphate buffer of pH 6.5, which was identical with the solution used for suspending the virus. The stock albumin solution had a density of 1.0686 and the results of viscosity measurements[1] made on it at 25° gave the data shown in Fig. 1. The relative viscosity of the buffered saline solution, measured also at 25°, was 1.025.

For the studies on the relation of sedimentation velocity to virus con-

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[1] The measurements of viscosity were very generously made by Dr. John O. Erickson.

centration, the virus preparations were diluted to the desired virus content with appropriate volumes of the buffered saline solution.

The virus-albumin preparations for the density studies were made by diluting the stock virus suspensions with the buffered saline solution and mixing with the stock albumin solution described above. These mixtures contained, in all instances, 2 mg. of virus per ml. and the respective amounts of bovine serum albumin required to vary the density from 1.0083 (the density of the buffer-saline solution) to 1.0686 in a series of five steps. It was necessary to mix fairly small volumes of the virus preparations in order to conserve the supply. Small total samples of 0.8 to 1.0 ml. could not be made up by pipette from the three ingredients with sufficient accuracy for the work and, consequently, the mixtures were made by weighing to 0.1 mg. the stock solution of albumin of previously. measured density. Virus in buffered saline was added and the total sample weighed to 0.1 mg. The density of the suspending medium surrounding the virus particles was

FIG 2   A series of typical schlieren photographs showing the progress, at 3 minute intervals, of the papilloma virus boundary as it moves down the ultracentrifuge cell. The radial distances measured to the different boundary positions yielded data for analysis such as that shown in Fig 3. The concentration of virus was 3 mg per ml.

then calculated from these values. To prepare the sample containing the highest concentration of albumin, the virus was sedimented in the ultracentrifuge and resuspended in the undiluted albumin solution. When a sample was prepared, about half of it was placed in the rotor cell of the ultracentrifuge immediately, and its sedimentation characteristics were recorded. Most of the remainder of the sample was similarly studied the following day, and the residue was employed for pH measurement.

Sedimentation velocity measurements were made in an air-driven ultracentrifuge carrying a 4° sector-shaped cell 12 mm. high at a mean radius of 6.5 cm. The cell thickness was 5 mm. Photographic record of the sedimenting boundary was made by virtue of its accompanying refractive index gradient. Schlieren pictures were taken with a lens system giving 2 × magnification in the direction of sedimentation. Because of the high degree of homogeneity of the papilloma virus and the short time of sedimentation, little change in the position of the "schlieren cutter" was necessary during the runs. In Fig. 2 there is shown a representative series of

photographs[2] made at 3 minute intervals while the rotor turned 210 R.P.S. Measurements to locate the boundary positions on the negatives were made with a traveling microscope equipped with low power lenses.   In each case the mid-point of the shadow was taken as the position of the boundary. Common logarithms of the radii obtained from this plate are shown in Fig. 3 plotted against time, giving the line whose slope was used for sedimentation velocity calculations as follows:

$$\frac{2.303(\log_{10} R_2 - \log_{10} R_1)}{60\omega^2 (T_2 - T_1)} \qquad K(\text{slope}) \qquad (1)$$

where R_1 and R_2 are radial distances in cm. to the boundary at times T'_1 and T_2 measured in minutes; ω is the angular velocity; and K is the constant for a given rotor speed.
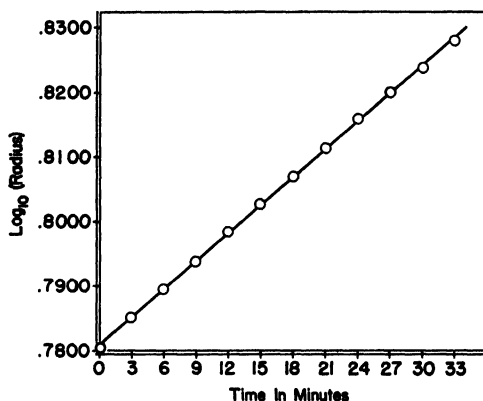
FIG. 3.   Analysis of a series of schlieren photographs showing the $\log_{10}$ of the radial distances plotted against time.   The slope of the line is proportional to the sedimentation rate.

In all of the determinations, care was taken to have the temperature of the sample the same as that of the rotor.   The temperature of the rotor was measured before and after each run to provide a mean value from which density and viscosity corrections were calculated.

Results

The findings concerned with the dependence of sedimentation velocity on virus concentration are illustrated in Fig. 4.   The sedimentation constants ($S_{20°}$) of a single virus preparation at various virus concentrations fall on a straight line inclined only slightly to the horizontal, showing a

[2] These photographs supplied the data for the point of Fig. 4 corresponding to 3 mg. of virus per ml.

small but definite effect of concentration on S_{20}, for this preparation. The results with a second preparation at two different concentrations are also shown in Fig. 4. The straight line was drawn through all of the points by the method of least squares. From these results, it appears that little change occurred in S_{20}, of the papilloma virus at concentrations up to 6 mg. per ml. The value of S_{20}, used for estimation of particle radius in subsequent calculations was obtained by extrapolating the relation to zero virus concentration. This value was S_{20}, = 297 Svedberg units.

The results of the studies on density of the virus particles in the bovine serum albumin are given in Figs. 1 and 5. In Fig. 1 there are shown the observed sedimentation rates, multiplied by the absolute viscosity of water, η_t^0, at the temperature of the run in relation to the density of the albumin solutions. It is seen that the sedimentation rate of the virus decreases very rapidly with the increase in the density of the albumin
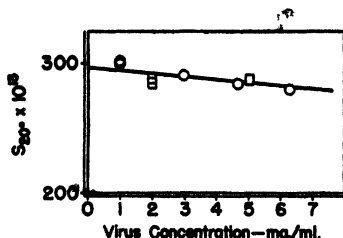
FIG. 4. The dependence of sedimentation constant (S_{20},) on papilloma virus concentration is shown extrapolated to infinite dilution, indicating a limiting value of the sedimentation constant of 297 Svedberg units. The open circles represent the sedimentation constants of a single preparation at various virus concentrations. A second preparation at two different concentrations is shown by the square symbols.

solution. The increase in the viscosity of this suspending medium associated with increase in the concentration of albumin is also indicated in Fig. 1. It is seen, further, that at the higher densities of the media the virus sedimented at speeds ($\eta_t^0 S = (28 \times 10^{-15})$) so low that the sedimentation rate of bovine albumin itself ($\eta_t^0 S_A$ = about 5×10^{-15}) at the temperatures of the studies contributed significantly to the observed rate of sedimentation of the virus. Thus, if the rotor speed and albumin concentration could be increased indefinitely, the rate of virus sedimentation would never reach zero but would approach asymptotically that of the albumin. This approach would be exceedingly slow because of the rapid increase in the viscosity of the albumin solution through which the virus must sediment.

In order to estimate the influence of the density of the suspending medium alone on the sedimentation rate, therefore, two corrections must be made. First, the sedimentation velocity of bovine serum albumin must be cal-

culated from its sedimentation constant at each temperature used and sub-tracted from the observed velocity of the sedimenting virus boundary; and, second, this difference must be multiplied by the relative viscosity of the suspending medium (assumed in this experiment to be constant over the temperature range used, namely 23.4–27.0°),[3] and by the absolute viscosity of water at the temperature of the experiment.

The resulting sedimentation velocity values corrected as described are given in Fig. 5, plotted against the density of the suspending medium. One set of the values was obtained from the results of examination of the virus-albumin mixtures immediately after preparation and another group from examination of the same mixtures after 24 hours. There was no evidence
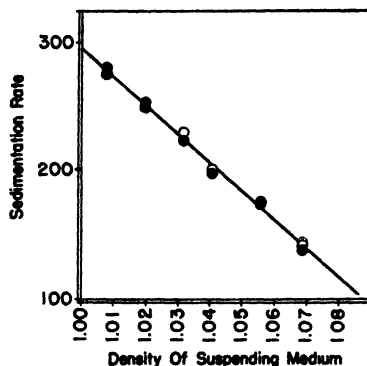
FIG. 5. The dependence of sedimentation rate of the papilloma virus on the density of the bovine albumin suspending medium. Correction of the data of Fig. 1 for the effects of viscosity and finite sedimentation of the albumin yielded the linear relation-ship shown here. These data indicate a virus particle density of 1.133 under the conditions of the experiment. The closed circles represent the results obtained immediately after preparation of the virus-albumin mixtures; the open circles, the results for the same mixtures after 24 hours.

of systematic influence of time, and the straight line shown was con-structed through all of the points by the method of least squares. The results reveal no evidence of any consistent deviation of the points from the linear relationship. It may be seen that the accuracy of repeated estimates on a given preparation is definitely greater than that from preparation to preparation, as judged from the closeness of points to each other and to the line. Residual weighing errors may account for this.

The sedimentation rate of a particle is determined by the strength of the acceleration field in which it lies, by the increment of unbalanced mass after buoyancy allowance has been made, and by the frictional resistance

[3] The assumptions involved in these corrections lead to errors smaller than the ran-dom errors of the experiments.

to sedimentation offered by the fluid through which movement is imminent. The sedimentation constant, as defined by Svedberg for protein molecules, is the sedimentation rate corrected to the density and viscosity of water at $20°$ ($S_{20°}$). These corrections are applied as follows:

$$S_{20°} = S \left(\frac{\eta_t^0}{\eta_{20°}^0} \right) \left(\frac{\eta_{s_i}}{\eta_t^0} \right) \left(\frac{1 - \rho_{20°}^0 \, V_{20°}}{1 - \rho_t \, V_t} \right) \tag{2}$$

where the symbols have the following meanings: $\eta_t^0 =$ the viscosity of water at the temperature of the centrifuge run; $\eta_{20°}^0 =$ the viscosity of water at $20°$; $\eta_{s_t} =$ the viscosity of the suspending medium at the temperature of the centrifuge run; $\rho_{20°}^0 =$ the density of water at $20°$; $\rho_t =$ the density of the suspending medium at the temperature of the centrifuge run; $V_{20°} =$ the partial specific volume of the virus at $20°$; $V_t =$ the partial specific volume of the virus at the temperature of the centrifuge run; and $S =$ the observed sedimentation rate.

This is a convenient form of the equation, for the first parentheses hold the temperature correction for the viscosity of water which varies from run to run; the second hold the correction for relative viscosity of the buffer solution, which is constant over a fairly great temperature range; and the third hold the correction for buoyancy. The relationship between the fundamental quantities influencing sedimentation can be indicated as follows:

$$\text{(Particle volume)} \quad (\rho_v - \rho_t) \, \omega^2 R = f \frac{dR}{dt} \tag{3}$$

where $\omega^2 R$ is the centrifugal field strength; the particle volume times the difference between the density of the virus (ρ_v) and that of the suspending medium (ρ_t) is the unbalanced mass; and f is the frictional resistance per unit velocity dR/dt in the R direction. If the particle is a sphere, as indicated by electron micrographs (10), then its volume is known in terms of its radius. Its frictional coefficient, according to Stokes, is known also in terms of its radius, r. The relationship (Equation 3) now becomes

$$\frac{4}{3} \pi r^3 \, (\rho_v - \rho_t) \omega^2 R = 6\pi \eta_{s_i} r \frac{dR}{dt}$$

therefore,

$$\frac{2}{3} r^2 (\rho_v - \rho_t) = 3\eta_{s_i} \frac{1}{\omega^2 R} \frac{dR}{dt} = 3\eta_{s_i} S$$

by definition of S,

$$= 3 \sqrt{\frac{\eta_{s_t} \, S}{2(\rho_v - \rho_t)}} \tag{4}$$

The linear relationship between $\eta_{s_t}S$ and ρ_t or $(\rho_v - \rho_t)$ predicted by these equations is borne out in the present experiments, as is shown in Fig. 5. Assuming that particle size, shape, and density of the virus are unaltered as the bovine albumin density around it changes (a condition suggested but not proved by the linearity of the relationship), the virus density calculated from these data is 1.133 for the region under investigation. In order to obtain virus particle size, the value of $S_{20°}$ at infinite virus dilution, Fig. 4, is necessary and yields, with appropriate substitution in Equation 4, the value of the radius $r = 33 \times 10^{-7}$ cm., or 33 mμ.

If the virus particle is thought to be composed of a substance whose dry density is $1/V$ and some quantity of water, this quantity can be calculated from V and ρ_v. The partial specific volume of the papilloma virus determined in previous work (11) was 0.754; pycnometric measurements on the material employed in the present studies gave 0.761. When the value 0.761 is used, the calculated amount of water associated with the virus particle is 58 per cent by volume, or 1.04 gm. of water per gm. of dry virus. If the liquid part of the sedimenting virus unit is not water but buffer salt solution ($\rho = 1.0083$), the value is 59 per cent by volume.

DISCUSSION

In the present studies, the sedimentation velocity of the papilloma virus in bovine serum albumin solutions, corrected for known conditions of the experiment, was found to be related in a linear fashion to the density of the suspending albumin solution. With the data thus obtained, the density of the virus particles was found to be 1.133 in the region investigated. With respect to the character of the relationship between density of the suspending medium and the sedimentation velocity of the virus, the results with the papilloma virus were analogous to the findings with influenza viruses A and B and the swine influenza virus (5, 6). In the work with the papilloma virus, greater accuracy in estimating sedimentation velocity was necessary in order to attain a comparable degree of precision in the final value of density because the slope of the line, ηS versus $(\rho_v - \rho_t)$, for the papilloma virus is less than the analogous slopes for the influenza virus. The smallness of size and the relatively great density of the papilloma virus account for this and necessitate the weighing of samples and correction for appreciable sedimentation of the bovine serum albumin itself.

The density value, 1.133, considered with the partial specific volume 0.761, reveals the presence of a large amount of water associated with the papilloma virus particle, and the diameter of a spherical particle of this sort (10), 66 mμ, is much greater than the size, 44 mμ, observed from electron micrographs (10). Removal of the 58 per cent water, if it were accompanied by 58 per cent shrinkage in the volume of the particle without

change in shape, would reduce the diameter of the particle to 48 mμ. The significance of such a value, however, is questionable, for the shrinkage, if any, might not be as much as 58 per cent. In the initial measurements in electron micrographs it was realized (10, 12) that the values for the diameter might be low because of the obvious diffuseness in particle limits. It is possible that the difference between the values 66 and 44 mμ may be associated with both difficulty in measurement in electron micrographs and shrinkage of the particle on drying. No evidence has been found for shrinkage of bacteria (13), but the diameters of influenza virus particles observed in electron micrographs (6) are generally significantly lower than those calculated from observed density and sedimentation velocity data.

In previous work (11) direct measurements were made on the rate of diffusion of a preparation of papilloma virus which was characterized by a sedimentation constant of 280 $\times$ 10^{-13} and a dry density of 0.754. The diffusion constant of the material was 6.65 $\times$ 10^{-8}. Markham, Smith, and Lea (14), in calculations from the above data together with the electron micrographic evidence of the virus particle shape (10), predicted the value 73.4 mμ for the particle diameter and 1.88 gm. of water in association with 1 gm. of the dry virus. From the data of the present work, the diameter of the particle in aqueous media was 66 mμ, the amount of associated water 1.04 gm. per gm. of dry virus, and the calculated diffusion constant 7.2 $\times$ 10^{-8} cm.^{2} per second. On consideration of the many factors possibly involved, the two sets of data and calculated values are not necessarily inconsistent. Measurement of diffusion constants, especially of such large particles, may be subject to considerable variation. It should be noted that small differences in the rate of diffusion of particles in this range of size and degree of hydration reflect relatively great differences in both size and water content. The same is true, also, for variation in the values of the sedimentation constant, but measurements of sedimentation velocity for this size range are of far greater accuracy than diffusion measurements. There is reason to believe, however, that the differences between the previous data and those of the present work may reflect, at least in part, actual variation in the characters of the papilloma virus from one preparation to another. It is well to recall that the sedimentation velocity studies of the previous work (11) were made on twelve different purified virus preparations and that the values observed varied from 266 to 288 about a mean value of 278.3 Svedberg units. Such variation is well outside the limits of error of the method of measurement and might be accounted for either on the basis, possibly, of dissymmetry in shape or, more probably, of the amount of water associated with the particle. It is unlikely that the chemical constitution of the virus is subject to great differences, since the values of dry density, 0.754 for the material of the previous work and 0.761

for that used here, show small variation.   If the papilloma virus is assumed
to be molecular, variation in experimental data is difficult to explain. ⌐ On
the contrary, variation among living organisms is to be expected.

The interpretation of the data relative to diffusion, size, and shape of
the papilloma virus previously obtained (11) obviously was greatly in
error.   These interpretations were based on the judgment that the pre-
ponderance of evidence then available indicated that the papilloma virus
was a molecular nucleoprotein.   Recent experience in the study of animal
viruses (12), especially with the electron microscope, renders this position
untenable at the present time.   Electron micrographic studies on the
influenza (15–17), vaccinia (18), and equine encephalomyelitis viruses (19)
have revealed variations in structure, shape, or size which definitely remove
these agents from the category of molecular materials.   Because of the
low contrast in the electron micrographic images of the papilloma virus
(10), unequivocal evidence of morphological variation in the papilloma
virus has not yet been obtained.   The conclusion cannot be evaded, how-
ever, that the properties and behavior of the papilloma virus revealed in
electron micrographs and in the present work are much more closely similar
to those of the viruses mentioned above and to living matter in general
than to the characters expected of molecules.

SUMMARY

Studies were made on the sedimentation velocity of purified papilloma
virus in bovine serum albumin solutions of various concentrations and
densities.   The relation of sedimentation velocity to the density of the
medium was linear in the region studied when suitable corrections were
made for viscosity of the medium and the sedimentation of the albumin
molecules occurring in the centrifugal fields employed.   From this relation-
ship, calculations of the density of the virus particle in aqueous suspension
gave the value of 1.133.   The partial specific volume of the virus material
studied was 0.761, which is in satisfactory agreement with previous find-
ings, 0.754.   By extrapolation of the linear relationship of sedimentation
velocity to virus concentration, the sedimentation constant at infinite virus
dilution was found to be 297×10^{-13}.   From the sedimentation velocity
data and the density of the particle with associated water, the diameter of
the hydrated spherical particle was 65.6 mμ.   With the reciprocal of the
partial specific volume, 1/0.761, as the dry density and the value 1.133
as the wet density, the amount of water associated with the virus was
calculated to be 58 volumes per cent, or 1.04 gm. of water per gm. of dry
virus.   The calculated diffusion constant for particles of these characters
was 7.2×10^{-8}.

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A RAPID MICRODETERMINATION OF GLYCOGEN IN TISSUE SLICES*

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For the quantitative determination of glycogen in tissues a choice of methods is available. If the organ under investigation contains no other alkali-stable polysaccharides besides glycogen, the quantitative method introduced by Bernard (1) and improved by Pflüger (2) can be used. The pulped tissue material is treated with an equal volume of hot 60 per cent potassium hydroxide solution and precipitated with a like volume of alcohol. The precipitate is washed and the purified residue dissolved in water and hydrolyzed in hydrochloric acid. The resulting glucose is then determined by one of the available reduction methods (3–5). Sørensen and Haugaard (6) determined the resulting glucose by means of a colorimetric method with orcinol. Heatly (7) modified Pflüger's method so that as little as 1 mg. ± 2 γ could be determined. After the hydrolysis the reducing sugar was determined with the method described by Linderstrøm-Lang and Holter (8). Good, Kramer, and Somogyi (9) abbreviated Pflüger's method, and determined the glycogen after hydrolysis as reducing sugar by the method of Shaffer and Hartmann (3). Jung (10) described a colorimetric method for the determination of glycogen in large amounts of liver tissue (10 gm.), in which the color produced with iodine is used as a measure for the glycogen concentration. In all these methods except Jung's procedure, the glycogen is determined indirectly, and usually more than one transfer of the solutions is necessary.

For some of our studies on the tissue metabolism of guinea pigs deficient in the antistiffness factor (11) it was necessary to determine the glycogen present in tissue slices before and after the experiment. Since only a maximum amount of 75 mg. of tissue was available for the determination, a rapid direct colorimetric method requiring as little transfer as possible would be of great advantage. We have developed a micro colorimetric method adapted to the Klett-Summerson colorimeter with which the glycogen present in slices weighing approximately 50 mg. could be accurately determined. Only one transfer is necessary, since the precipitation

of the glycogen and the subsequent developing of the color are carried out in the same colorimeter tube. In our hands the method has given good results and satisfactory recovery values could be demonstrated.

<div align="center">EXPERIMENTAL</div>

Reagents—

1. *Potassium hydroxide solution, 35 per cent.* 350 gm. of potassium hydroxide are made up to 1 liter in boiled (CO_2-free) distilled water. The solution is stored in a Pyrex bottle equipped with a soda lime tube.

2. *Ethanol, 95 per cent.*

3. *Hydrochloric acid, concentrated.*

4. *Phenolphthalein,* 1 per cent solution in 50 per cent ethanol.

5. *Lugol's solution.* 1 gm. of iodine is dissolved in a solution containing 2.0 gm. of potassium iodide in 20 cc. of water. The solution is kept in a well stoppered dark bottle.

6. *Stock glycogen solution.* 25 mg. of glycogen[1] (Eastman Kodak Company, White Label) are dissolved in 25 cc. of a 35 per cent potassium hydroxide solution. The solution keeps well if stored at room temperature.

<div align="center">*Method*</div>

A tissue slice (liver, kidney) or muscle strip, weighing from 50 to 75 mg., is dropped into 2 cc. of 35 per cent potassium hydroxide solution contained in a 6 inch Pyrex test-tube which is equipped with an air condenser. The tube is placed in a boiling water bath and the contents are refluxed for 2 hours. At the end of this period, the digest is filtered through starch-free filter paper (Whatman No. 41) into a calibrated colorimeter tube (Klett Manufacturing Company, Inc.) in which a crystal of potassium iodide is placed. The residue on the filter paper is washed with 1 cc. of distilled water. The filtrate is made up to 10 cc. with 95 per cent ethanol, 1 drop of indicator is added, and the contents of the tube are thoroughly mixed by lateral shaking. The solution is then neutralized with concentrated hydrochloric acid. After the mixture has cooled to room temperature, 1 drop of hydrochloric acid is added in excess to coagulate the glycogen, a process which is completed in about 3 minutes. The tubes are then centrifuged for 5 minutes at 3000 R.P.M. in an angle centrifuge. The supernatant liquid is decanted and the precipitated glycogen is dissolved in 1 to 2 cc. of warm distilled water. This solution is diluted to the 5 cc. mark and exactly 0.05 cc. of Lugol's solution is added from a micro measuring pipette. The contents of the tube are mixed well by

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[1] The glycogen used for the standard should be dried to constant weight and its purity determined according to one of the standard methods (9). The glycogen used for our standard was 98.4 per cent pure.

inversion and the color is read immediately in the colorimeter equipped with a No. 54 filter. The blank reading is given by a solution containing 5 cc. of distilled water and 0.05 cc. of Lugol's solution. A reading of the standard is included in each series of determinations. This standard is prepared by diluting 1 cc. of the stock solution with 1 cc. of 35 per cent potassium hydroxide solution. This mixture is then carried through the same procedure as that described above for the precipitation and color
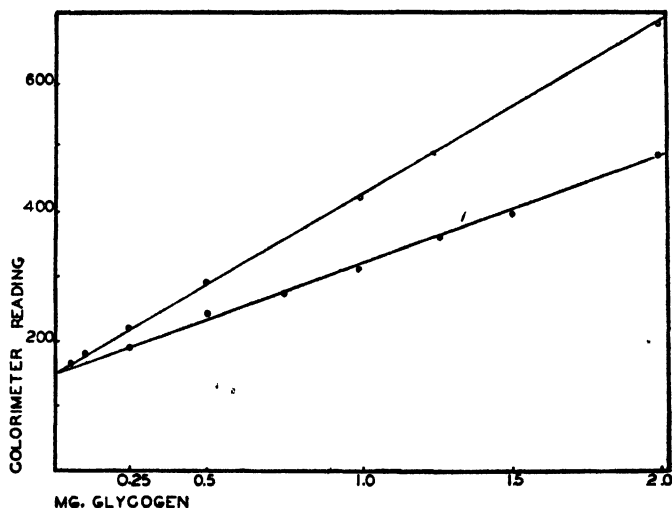

FIG. 1. Relation between glycogen concentration and colorimeter readings. Upper curve, glycogen dissolved in potassium hydroxide solution (see the text); lower curve, glycogen dissolved in distilled water. The points are averages of triplicate determinations.

development in the actual glycogen determination in tissue slices. The glycogen content of the sample is calculated from the formula,

$$\% \text{ glycogen} = \frac{(\text{reading of sample}) - (\text{reading of blank})}{(\text{reading of standard}) - (\text{reading of blank})} \times \frac{100}{\text{weight of sample}}$$

or read from the calibration curve.

Results

It was found that the colored solution follows Beer's law if the concentration of the glycogen ranges from 0.05 to 2 mg. per 5 cc. (Fig. 1). A lower color density results when the glycogen is dissolved in water and directly treated with Lugol's solution. Varying concentrations of potas-

TABLE I

Glycogen Determinations at Various Levels of Liver Glycogen

Liver source	Liver	Glycogen found	Glycogen
	mg.	*mg.*	*per cent*
Normal guinea pig	71.6	11.69	16.3
	72.0	12.31	17.1
	80.8	13.59	16.8
	104.0	15.10	14.5
	123.9	19.43	15.7
	127.0	23.50	16.3
Mean ± standard error....................................			16.1 ± 0.3
Guinea pig fasted for 24 hrs.	44.6	0.29	0.63
	47.7	0.37	0.68
	55.4	0.38	0.68
	56.2	0.37	0.66
	81.6	0.48	0.59
Mean ± standard error.............			0.65 ± 0.02

TABLE II

Recovery of Glycogen Added to Liver Slices

Liver tissue	Glycogen in tissue (a)	Glycogen added (b)	Total glycogen found (c)	Glycogen recovered (c − a)	Recovery
mg.	*mg.*	*mg.*	*mg.*	*mg.*	*per cent*
51.2	0.12	1.00	1.13	1.01	101
60.8	0.15	1.00	1.10	0.95	95
61.4	0.15	1.00	1.07	0.93	93
52.1	0.12	1.00	1.11	0.99	99
64.8	0.16	1.00	1.06	0.91	91
48.6	0.12	1.00	1.20	1.07	107
81.3	2.90	1.00	4.00	1.10	110
67.5	2.45	1.00	3.43	0.98	98
96.4	0.44	0.50	1.00	0.56	112
89.0	0.25	0.50	0.80	0.55	110
Average..					101.6

sium iodide added to the solution of glycogen in distilled water did not increase the color intensity. Only after the glycogen is subjected to the alcohol treatment does the·color deepen. It is because of this difference

in behavior that the stock solution of glycogen is made up in the strongly alkaline solution and that the standard determination is carried through the same procedure as the glycogen determination in tissues. The difference in the intensity of the iodine color of glycogen dissolved in water compared to glycogen after precipitation with alcohol is probably due to the inclusion of potassium chloride in the precipitate. This, however, should not interfere with the accuracy of the method, since the same amounts of potassium hydroxide, hydrochloric acid, and alcohol are always used in each determination.

In Table I the concentrations of glycogen in the livers of guinea pigs on a stock diet and of guinea pigs which had been fasted for 24 hours before the experiment are presented. It will be noted that the standard errors are small.

TABLE III

Comparison of Colorimetric Method with Glycogen Determination according to Good et Al.

Good, Kramer, and Somogyi method		Colorimetric method	
Liver	Glycogen	Liver	Glycogen
gm.	*per cent*	*gm.*	*per cent*
1.253	2.97	0.1000	3.17
1.052	3.01	0.1250	2.98
1.165	3.15	0.1014	3.13
Average	3.04		3.09

Glycogen values found with the proposed method cannot be considered valid unless satisfactory recovery values of added glycogen can be obtained. It can be seen from Table II that the recovery of amounts of glycogen ranging from 0.5 to 1 mg. are within the experimental error.

The determination was compared with that according to Good *et al.* and good agreement was found (Table III).

SUMMARY

A rapid colorimetric determination of glycogen in tissue slices is described. The method makes use of the color produced when glycogen is treated with iodine. Satisfactory recovery values can be obtained. Good agreement exists between the values obtained by the determination of glycogen according to Good, Kramer, and Somogyi and by the proposed method.

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STRUCTURAL REQUIREMENTS FOR DIABETOGENIC ACTION IN ALLOXAN AND RELATED COMPOUNDS

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Investigators have been unable to alter the structure of alloxan without loss of diabetogenic activity. Related compounds reported to be without action on the islet tissue of the pancreas or on blood sugar are mesoxalic acid (1), formylurea (1), formyloxaluric acid (1), parabanic acid (1, 2), uramil (2), oxaluric acid (1), violuric acid (2, 3), dialuric acid (1, 3), isodialuric acid (1), isobarbituric acid (1), alloxanic acid (1), barbituric acid (1, 3), and benzalbarbituric acid (2).

The purpose of this investigation was to prepare, and test for diabetogenic action, several compounds not already reported in the literature related either by structure or chemical properties to alloxan, with the object of determining, if possible, the particular portions of the alloxan molecule responsible for the diabetogenic action.

EXPERIMENTAL

Table I summarizes the materials tested. Sprague-Dawley and Wistar albino rats weighing from 120 to 250 gm. were used. Water-soluble compounds were dissolved in water; those insoluble in water were either dissolved or suspended in propylene glycol. Both subcutaneous and intraperitoneal injections were tried in most cases. The animals were kept in metabolism cages and were fed a diet of commercial dog chow. All animals were observed for a period of at least 5 days after injection and the urine was examined qualitatively (Benedict's test) for the presence of sugar. Since the purpose of the work was to establish the efficacy of the compounds tested in producing a permanent diabetes mellitus, blood sugar determinations were done only when glycosuria developed or when animals appeared to be in a condition suggesting hypoglycemic shock. All blood sugar determinations were done according to the method of Folin and Malmros (9).
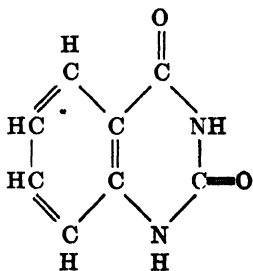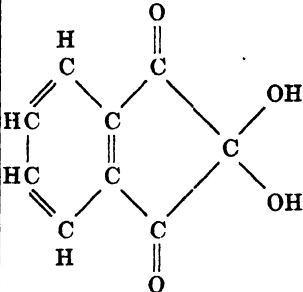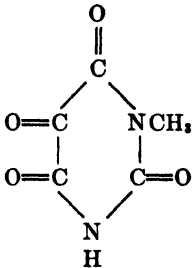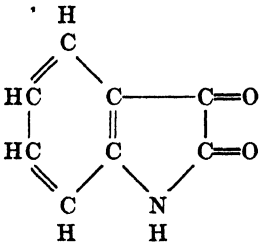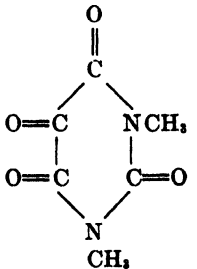
Table II summarizes the results obtained with the compounds listed in Table I.

DISCUSSION

Negative results obtained with sodium mesoxalate, ethyl mesoxalate, and mesoxalamide serve to substantiate the earlier work, mentioned above,

which indicated that the intact pyrimidine ring is essential for diabetogenic activity.

<div align="center">

TABLE I

Summary of Compounds Tested

</div>

Compound tested	Structure	Compound tested	Structure
Sodium mesoxalate (4)*	NaOOCCOCOONa	Benzoylene urea (7)	
Ethyl mesoxalate (4)	$C_2H_5OOCCOCOOC_2H_5$		
Mesoxalamide (5)	$H_2NOCCOCONH_2$		
Ninhydrin†		Monomethyl alloxan (8)	
Isatin (6)		Dimethyl alloxan (8)	

* The figures in parentheses are bibliographic reference numbers.
† Eastman Kodak Company.

Alloxan shares with triketohydrindene (10) and isatin (11, 12) the property of oxidative deamination of α-amino acids with the production of aldehydes containing 1 less carbon atom (13, 14). With the idea in mind that the actual diabetogenic agent might be one of these aldehydes, ninhydrin

and isatin were tested.   Ninhydrin, as expected, proved highly toxic, but blood sugar determinations failed to show any permanent alteration of the glucose concentration, although a slight transient hyperglycemia was noted in some rats.[1]   Isatin had no activity and did not appear to be toxic in the

TABLE II

Summary of Experimental Conditions and Results

Compound tested	No. of animals	Dose	Concentration	Results
		mg. per kg.	*per cent*	
Sodium mesoxalate	12	30–800*†	1 –5	None
Ethyl "	3	326*	5	"
Mesoxalamide	12	300–600†‡	2.5 –5	"
Isatin	6	60–200†	0.67	"
Triketohydrindene hydrate (ninhydrin)	44	35–300*	0.5 –5	Slight transient hyperglycemia; toxic in doses greater than 40–50 mg. per kilo
Benzoylene urea	12	25–400*‡	1 –2	Mild anesthesia, no blood changes
Monomethyl alloxan	10	200–250†	3	Strong glycosuria in 18 hrs.; permanent hyperglycemia in all animals
Dimethyl alloxan	23	150–300*†	1 –3	Produced death in doses over 150 mg. per kilo without alteration of blood sugar; no blood sugar changes from 150 mg. per kilo

* Intraperitoneally.
† Subcutaneously.
‡ In propylene glycol.

dosages tried.   This evidence would indicate that the reactions of alloxan involving similar oxidations are not responsible entirely, if at all, for the

[1] It was suggested that injected ninhydrin present in the blood stream reacting with the Folin-Malmros ferricyanide-cyanide reagent might account for the observed hyperglycemia. To determine whether this might be true, blank determinations were run on ninhydrin solutions containing a quantity of ninhydrin equivalent to that which would be present in 0.1 ml. of whole blood after the injection of 35 mg. per kilo. Although the route of injection was intraperitoneal, it was assumed that the entire quantity might be present in the blood stream after 1 hour. For purposes of calculation, a blood volume of 50 ml. per kilo was used. Such quantities of ninhydrin gave a blank color equivalent to about 16 mg. per cent of sugar. The average elevation of the blood sugar of seven rats 1 hour after injecting 35 mg. of ninhydrin per kilo was 42 mg. per cent. It is felt that although the suggested reaction of ninhydrin with the sugar reagent might account for a small part of the rise noted it could not represent more than a few per cent of the increase.

diabetogenic activity of that compound. The fact that dialuric acid is incapable of producing a permanent hyperglycemia (3) may be taken as evidence that, although the oxidizing properties of alloxan do not play a direct part in the diabetogenic activity of that compound, at least it is essential that the molecule be in the oxidized form.

Seven animals receiving monomethyl alloxan developed a permanent hyperglycemia, while three died. Two of these three were given 250 mg. per kilo doses; the other received 200 mg. per kilo. Only one animal survived 250 mg. per kilo and at the end of 3 days had a blood sugar level of 666 mg. per cent. Blood sugar in animals receiving 200 mg. per kilo averaged 385 ± 45 mg. per cent. All these animals excreted acetone and acetoacetic acid. After 1 month, all were still excreting large quantities of sugar.

The striking difference in the actions of monomethyl alloxan and dimethyl alloxan may be attributable to the presence in the former (and alloxan itself) and the absence in the latter of a labile hydrogen atom at-

$$
\begin{array}{ccc}
& \text{O} & \\
& \parallel & \\
& \text{C} & \\
& \diagup \quad \diagdown & \\
\text{O}=\text{C} & & \text{N}-\text{CH}_3 \\
\mid & & \mid \\
\text{O}=\text{C} & & \text{C}=\text{O} \\
& \diagdown \quad \diagup & \\
& \text{N} & \\
& \text{H} &
\end{array}
\quad \rightleftharpoons \quad
\begin{array}{ccc}
& \text{O} & \\
& \parallel & \\
& \text{C} & \\
& \diagup \quad \diagdown & \\
\text{O}=\text{C} & & \text{N}-\text{CH}_3 \\
\mid & & \mid \\
\text{O}=\text{C} & & \text{C}=\text{OH} \\
& \diagdown \quad \diagup & \\
& \text{N} &
\end{array}
$$
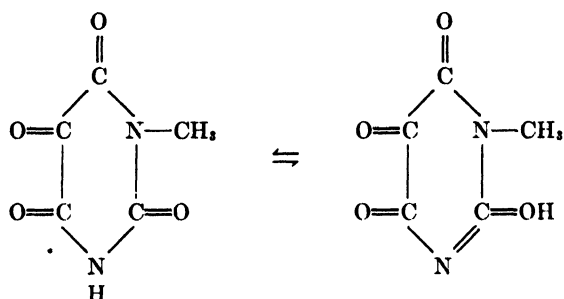
tached to nitrogen. Migration of this labile hydrogen to the oxygen of either adjacent carbon atom produces a compound containing 2 nitrogen atoms of differing basicity. Benzoylene urea, in which a similar system exists in regard to the nitrogen atoms, failed, however, to produce any changes resembling diabetes.

$$
\begin{array}{ccccc}
& \text{H} & & \text{O} & \\
& \text{C} & & \parallel & \\
& \diagup & & \text{C} & \\
\text{HC} & & \text{C} & & \text{NH} \\
\mid & & \parallel & & \mid \\
\text{HC} & & \text{C} & & \text{C}=\text{O} \\
& \diagdown & & \diagup & \\
& \text{C} & & \text{N} & \\
& \text{H} & & \text{H} &
\end{array}
\quad \rightleftharpoons \quad
\begin{array}{ccccc}
& \text{H} & & \text{O} & \\
& \text{C} & & \parallel & \\
& \diagup & & \text{C} & \\
\text{HC} & & \text{C} & & \text{NH} \\
\mid & & \parallel & & \mid \\
\text{HC} & & \text{C} & & \text{C}-\text{OH} \\
& \diagdown & & \diagup & \\
& \text{C} & & \text{N} & \\
& \text{H} & &
\end{array}
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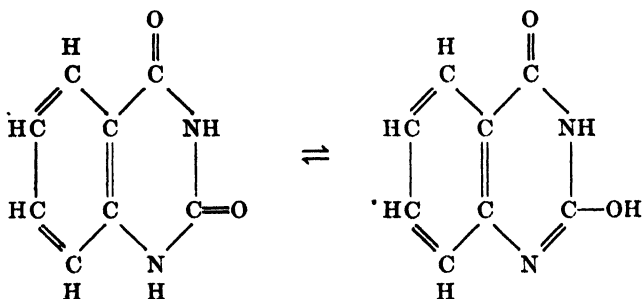
These pieces of evidence are particularly interesting in view of the fact that the only other compound at present recorded in the literature as possessing a selective necrotizing action on pancreatic islet tissue is 2(p-acetyl-

aminostyryl)-6-dimethylaminoquinoline methochloride (15), in which 2 nitrogen atoms of different basicity are joined by a system of conjugated double bonds.
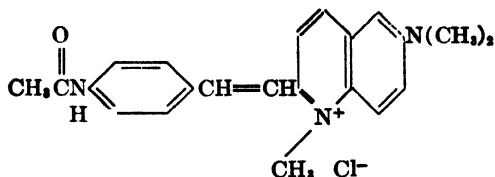
SUMMARY

Eight compounds related by structure or chemical reactions to alloxan have been tested for diabetogenic action on white rats.

Monomethyl alloxan is capable of producing experimental diabetes mellitus in white rats; neither dimethyl alloxan nor any of the other compounds tested was diabetogenic.

The significance of these results in relation to the structural requirements for diabetogenic action has been discussed.

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ON THE DETERMINATION OF PLASMA IODINE*

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During the past few years several methods for the estimation of micro amounts of iodine in biological materials have been developed. After the testing of several of these (1–3), the one selected for use in this laboratory was that published by Chaney in 1940 (3). With a few changes this procedure was found to yield satisfactory results for the determination of iodine not only in 3 cc. of plasma, but also in small amounts of thyroid tissue and in dietary mixtures.

In the present communication the results of various tests carried out to establish the reliability of the Chaney method as used in this laboratory are reported. The isotope of iodine, I^{131}, proved most useful in testing the method. Since its amount is determined only by a *radioactive* measurement, it provided a convenient and highly specific means for following the behavior of iodine in various steps of the procedure.

The method has also been employed to compare the plasma iodine levels of several common laboratory animals.

EXPERIMENTAL

Organic material is digested with a mixture of chromic and sulfuric acids, a process that oxidizes iodine to non-volatile iodic acid. The latter, together with the excess chromic acid, is reduced with phosphorous acid, and the volatile iodine formed thereby is distilled and trapped in an alkaline solution. The iodine is then measured by its catalytic effect on the reduction of ceric sulfate by arsenious acid.

Preparation of Reagents—

Distilled water. Double distilled water was used in the preparation of all solutions described below. Laboratory-distilled water was redistilled in an all-glass still in the presence of KOH.

70 per cent sulfuric acid. Sulfuric acid was freed of traces of iodine by adding 10 cc. of concentrated HCl to 2 liters of concentrated sulfuric acid and boiling the mixture for 1 to 2 hours. 195 cc. of the acid were added to 150 cc. of distilled water.

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0.15 N arsenious acid in 1.5 N H₂SO₄. 3.71 gm. of As_2O_3 were dissolved in a strong aqueous solution containing 2.5 gm. of NaOH. The solution obtained was then diluted to a volume of 250 to 300 cc. and neutralized with purified concentrated H_2SO_4. This was followed by the addition of 21 cc. of purified concentrated H_2SO_4, and the solution made up to a final volume of 500 cc.

Iodide solutions. A KI solution that contained 10.0 γ of I per cc. was freshly prepared every few months and stored in a dark bottle. From it were prepared the dilute standard solutions used in each measurement; the dilute solutions were renewed every few weeks.

50 per cent phosphorous acid. This was prepared by adding 100 cc. of water to 100 gm. of crystalline phosphorous acid. The crystalline product obtained from the General Chemical Company was tested and found to be satisfactory without further purification.

The following solutions were prepared in the manner described by Chaney (3): *60 per cent solution of chromic acid, 0.1 N ceric ammonium sulfate, and 1 per cent solution of NaOH.*

Procedure

3 cc. of plasma were used in all determinations. When total iodine was measured, this amount of plasma was added directly to the digestion mixture. In the determination of protein-bound iodine, the protein of 3 cc. of plasma was precipitated with zinc hydroxide as described by Man *et al.* (4). The precipitate was separated by centrifugation and washed two or three times with 20 cc. portions of distilled water.

Digestion—Plasma was digested with a mixture of 25 cc. of 70 per cent H_2SO_4 and 2 cc. of 60 per cent chromic acid in a two-neck, 300 cc. round bottom flask provided with interchangeable ground glass joints (3). In the determination of total iodine, 3 cc. of plasma were added directly to this digestion mixture. When protein-bound iodine was measured, the washed protein precipitate was first dissolved with 15 cc. of the H_2SO_4 and transferred quantitatively with an additional 10 cc. of 70 per cent H_2SO_4 to the digestion flask, which already contained the 2 cc. of chromic acid. The flasks were heated with 250 watt electric heaters and the contents agitated at the start of the digestion. Carborundum chips were found to be quite effective in promoting smooth boiling. The digestion was allowed to proceed until the flasks were fairly well filled with fumes; this occurred, as a rule, 1 to 2 minutes after the onset of fuming. At this point the flasks were removed from the electric heaters and allowed to cool. When the digestion mixture was sufficiently cool, 15 cc. of double distilled water were added and a second digestion carried out, as above, in order to insure the removal of

all volatile material which might interfere in the subsequent colorimetric determination.

Distillation of Iodine—A special still of the type designed by Chaney was used (3). In order to facilitate the removal of the distillate, a stop-cock, as described by Talbot *et al.* (2), was attached to the trap.

The digestion mixture was allowed to cool and 25 cc. of double distilled water added. The flask was then connected with the distillation apparatus described above and placed on a 350 watt Cenco electric heater.

The dropping funnel was now inserted into the side neck of the flask and 3 cc. of phosphorous acid placed in it.

As soon as liquid began to condense in the trap of the still, 1.2 cc. of 1 per cent NaOH were introduced into the trap by way of the condenser, which was adjusted to permit delivery into the trap. The condensed liquid as well as the NaOH served to seal the trap, and as soon as this occurred the phosphorous acid was added slowly from the dropping funnel. It was found advisable, as suggested by Chaney (3), to blow the acid into the flask in order to prevent loss of iodine by way of the stop-cock of the dropping funnel. For the first 30 seconds the condenser was turned to permit the condensate to enter the trap, a procedure that served to collect in the trap iodine that may have reached the condenser during this interval. The condenser was then adjusted so that the condensate returned to the boiling flask. The distillation was found to be complete in 5 to 7 minutes; the standard procedure adopted in all determinations was to distil for 7 minutes. At the end of the distillation period the contents of the trap were run through the stop-cock into a graduated 15 cc. centrifuge tube. The still was washed with a minimum of water; the total volume of distillate plus washing usually amounted to 13 to 14 cc.

Colorimetric Determination of Iodine—The determinations were carried out with a Klett-Summerson photoelectric colorimeter with the blue filter, No. 42. 4 cc. aliquots of the distillates were taken for the determination. To these were added 1 cc. of distilled water, 0.4 cc. of arsenious acid reagent, and, at a specified interval before the colorimetric readings were to be made, 0.3 cc. of ceric sulfate reagent.

Two blank samples were digested and distilled with each set of determinations for the preparation of the standard curves. The distillates from such samples were assumed to contain all the contaminating iodine present in the reagents as well as other substances which might affect the course of the colorimetric reaction. The two blank distillates were pooled and 4 cc. aliquots delivered into each of five colorimeter tubes. To each tube was then added 1 cc. of one of the dilute standard iodide solutions that contained amounts of iodine varying from 0 to 0.10 γ. Each tube thus served as a

single point for the standard curve. These standard samples were equal in volume to the unknown and had been obtained by treatment similar to that of the unknown. In this manner the standard curves were corrected for the reagent blank. Standard curves obtained on different days usually agreed within 10 per cent.

After the addition of 0.4 cc. of arsenious acid reagent to all samples, the colorimeter tubes were placed in a suitable rack and transferred to a bath maintained at $30° \pm 0.1°$. Since the color to be measured changes rapidly with time, it was necessary to read the color of each sample at a definite interval after the addition of the ceric sulfate. 0.3 cc. of ceric sulfate reagent was added to each tube at intervals of exactly 30 seconds. The ceric sulfate was accurately delivered by means of a syringe-pipette of the type described by Chaney (5). This interval allowed ample time for each tube to be shaken thoroughly after the addition of the ceric sulfate. Colorimetric measurements of each sample were made at exactly 10 and 20 minutes after the addition of the ceric sulfate. It was found convenient to read twenty tubes at one sitting; the 10 minute reading on the first tube was made immediately after the addition of the reagent to the last tube, and the 20 minute reading on the first tube immediately after the 10 minute reading on the last tube.

Test of Method

The protocol of a typical experiment with rat plasma is shown in Table I. Duplicate samples were taken from each distillate of plasma. The data show good agreement between the duplicate samples. The standard curves plotted from the data in Table I are shown in Fig. 1; for each plasma distillate the iodine values obtained by means of the 10 minute curve agreed very well with the values obtained from the 20 minute curve. The average of the two values was used in calculating the micrograms of iodine per 100 cc. of plasma.

Recovery of Added Iodine—In Table II are recorded the results of experiments in which the recovery of added iodine was determined with the aid of radioactive iodine (I^{131}). Radioactivity was measured by a scale of 8 Geiger-Müller counter. In Experiment 1 radioactive iodine[1] containing no measureable amount of I^{127} was added to 3 cc. of rat plasma before digestion; the recoveries of the added radioactivity ranged from 87 to 90 per cent. In Experiments 2, 3, and 4 both radioactive iodine (I^{131}) and 0.2 γ of I^{127} were added to 3 cc. of rat plasma. The average recovery of the I^{127} was 90 per

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[1] The radioiodine was prepared by bombarding tellurium with deuterons. The iodine separated contained the radioactive atoms plus the unavoidable traces of iodine present in reagents and glassware. Neither source of iodine is measurable by chemical analysis.

cent (84 to 94 per cent), that of the I^{131} 93 per cent (90 to 96 per cent). When the amount of I^{127} added (Experiment 5) was increased to 5 γ, its recovery amounted to almost 100 per cent.

From the results shown in Table I, the method used above for the determination of *plasma iodine* yields values that are 10 to 15 per cent low. But when this method is used only for comparing different samples of *plasma*, it is safe to conclude that comparisons are accurate to within 5 to 10 per

TABLE I

Protocol of Typical Determination of Iodine in Rat Plasma

Iodine standards	Rat No.	Volume of distillate (4 cc. aliquot for each determination)	Colorimeter reading		Iodine determined from Fig. 1		Plasma iodine
			10 min.	20 min.	10 min. average	20 min. average	
γ		*cc.*			γ	γ	γ *per 100 cc.*
0.0			540	500			
0.015			490	420			
0.030			440	340			
0.060			360	220			
0.10			260	103			
	1	12.9	450	355			
					0.028	0.028	3.0
			450	355			
	2	13.9	455	370			
					0.027	0.025	3.0
			450	360			
	3	13.3	440	345			
					0.030	0.029	3.3
			445	350			
	4	13.4	455	370			
					0.025	0.024	2.7
			460	370			
	5	13.2	435	335			
					0.033	0.032	3.6
			430	335			
	6	13.3	435	345	0.033	0.030	3.5

cent. For the determination of samples that contain more than 1 γ of iodine, the values obtained are probably no more than 5 per cent low.

To determine whether this loss of iodine occurred during digestion or during distillation, the following experiment was carried out: six 3 cc. portions of plasma were added to the chromic acid digestion mixture, and then to each sample was added 1 cc. of a solution containing radioactive iodine (I^{131}). These six samples were digested as described above. Two other plasma samples were first digested and 1 cc. of the I^{131} solution added there-

after. All samples were then made up to a volume of 2000 cc., and 1 cc. aliquots were taken for the determination of radioactivity. The amounts of the added radioactivity recovered in all eight samples were practically identical. This indicates that little, if any, loss of iodine occurs during
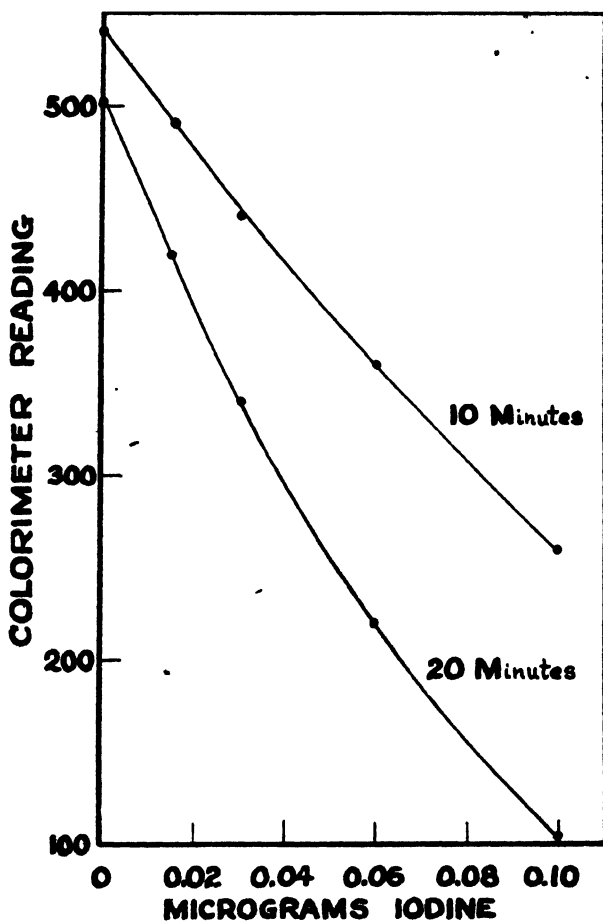
FIG. 1. Standard curves for plasma iodine determination

digestion and that the low values for plasma iodine referred to above result from a loss of iodine during the process of distillation.

The form in which the iodine volatilizes after iodate is reduced with phos-. phorous acid is not known with certainty. It is conceivable that the iodine might volatilize either as I_2 or as HI, or possibly in both forms. It was found here that, when radioactive iodine in the form of *iodide* was added

together with the phosphorous acid to a sample of digested organic material, the radioactive iodine was recovered almost quantitatively in the distillate. This observation is in agreement with the results of Fashena and Trevorrow (6), who reported that iodide added after reduction with phosphorous acid can be recovered in the distillate. These findings provide reasonable evidence that it is not necessary to reduce iodate only as far as I_2 in order to

TABLE II

Recovery of Added Radioactive and Chemical Iodine

Experiment No.	Material to which iodine was added	Sample No.	Iodine initially present	Iodine added as KI		Iodine found in distillate		Recovery of added iodine			
				Chemical	Radioactive	Chemical	Radioactive	Chemical		Radioactive	
			γ	γ	counts per sec.	γ	counts per sec.	γ	per cent	counts per sec.	per cent
1*†	3 cc. rat plasma	1		0	206		185			185	90
	3 " " "	2		0	206		179			179	87
	3 " " "	3		0	206		181			181	88
	3 " " "	4		0	206		182			182	88
	3 " " "	5		0	206		184			184	89
2	3 " " "	1	0.107	0.20	197	0.275	182	0.168	84	182	92
	3 " " "	2	0.107	0.20	197	0.279	178	0.172	86	178	90
	3 " " "	3	0.107	0.20	197	0.296	189	0.189	94	189	96
3	3 " " "	1	0.098	0.20	150	0.285	139	0.187	94	139	93
	3 " " "	2	0.098	0.20	150	0.283	141	0.185	92	141	94
	3 " " "	3	0.098	0.20	150	0.270	141	0.172	86	141	94
4	3 " " "	1	0.089	0.20		0.265		0.176	88		
	3 " " "	2	0.089	0.20		0.273		0.184	92		
	3 " " "	3	0.089	0.20		0.276		0.187	93		
5‡	200 mg. wheat	1	0	5.0	197		198			198	100
	200 " "	2	0	5.0	197		194			194	98
	200 " "	3	0	5.0	197		190			190	97

* Each of Experiments 1 to 4 was carried out on a single pool of plasma.
† Only radioactive measurements were made in Experiment 1.
‡ The wheat was iodine-free.

recover it quantitatively in the distillate. In the above experiments iodide as such (most probably as HI) was volatilized.

Separation of Inorganic Iodide from Protein-Bound Iodine[2] of Plasma—In order to test the separation of inorganic iodide from protein-bound iodine

[2] The term "protein-bound" refers to iodine that is precipitated along with proteins and that cannot be freed from the precipitate by washing with distilled water. It should not be inferred, however, that the iodine-containing compound or compounds of plasma are necessarily bound to the protein molecule by peptide linkages.

TABLE III

Separation of Inorganic Iodine from Protein-Bound Iodine of Rat Plasma

For a description of the experiment see the text.

Experiment No.*	Inorganic iodide added to 3 cc. of plasma	Protein-bound iodine, found before addition of iodide	Protein-bound iodine found after addition of iodide
	γ	γ per 100 cc.	γ per 100 cc.
1	1.0	3.2	3.2
		3.4	3.3
		4.1	
2	0.5	3.5	4.3
		3.1	4.7
			4.9
3	1.0	3.5	3.7
		3.1	4.5
			3.7
4	1.0	3.5	4.3
		3.1	4.5
		3.0	4.1

* Each experiment was carried out on a single pool of rat plasma.

TABLE IV

Protein-Bound and Total Iodine Levels in Plasma of Different Species

Animal	Condition of animal	Plasma, protein-bound iodine	Plasma, total iodine	Estimated iodine intake per day
		γ per 100 cc.	γ per 100 cc.	mg.
Dog	Postabsorptive	2.8	36	10– 15
"	"	3.1	14	10– 15
"	"		41	10– 15
"	"		52	10– 15
				γ
Chicken	Postprandial	3.6	8.4	90–100
"	"	3.8	6.4	90–100
"	"	3.3		90–100
Mouse	"	3.8	4.5	2– 3
"	"	3.8		2– 3
Rat	Postabsorptive	3.5	3.5	3– 4
".	"	3.4	3.3	3– 4
"	"	3.5	3.3	3– 4
Human	"	5.8	7.6	
"	"	5.7	7.4	
"	"	5.5	5.9	
"	"	7.8	6.7	
"	"	6.8	7.2	
"	"	6.8		

of plasma, either 1.0 γ or 0.5 γ of iodine in the form of KI (each contained in a volume of 0.1 cc.) was added to each of several 3 cc. samples of rat plasma. Each experiment (Table III) was carried out on a single pool of plasma obtained from several rats. The proteins in these samples were then precipitated with zinc hydroxide and separated by centrifugation. The precipitates were then washed three times, each with 20 cc. of distilled water. The values obtained for the protein-bound iodine of these plasma samples were compared with samples of the same plasma to which no inorganic iodide had been added. The data recorded in Table III show that three washings of the protein precipitate removed most of the added iodide. However, a small fraction of the added iodide probably remained in the protein precipitate, which would account for the somewhat higher values observed for protein-bound iodine in the presence of added inorganic iodide.

Iodine Content of Plasma

The total and protein-bound plasma iodine of rat, mouse, dog, chicken, and man was measured by the procedure described above; the values are recorded in Table IV. The values recorded for the daily iodine intake of each animal were based on an iodine analysis of their diet. Despite large differences in the levels of total iodine in plasma, the values obtained for protein-bound iodine of plasma of dog, rat, mouse, and chicken were quite similar. The values for protein-bound iodine of human plasma, however, were appreciably higher than those of the other species.

Although it was shown above that the Chaney method yields values for plasma iodine that are about 10 to 15 per cent low, the values recorded in Tables I to IV have not been corrected for this error.

DISCUSSION

Parts of the Chaney method for determining plasma iodine have been selected for use by other investigators (2, 7–9). Talbot et al. (2) employed the Chaney still, but combined it with a permanganate-ashing procedure and a photoelectric determination of a starch-iodine color. Salter et al. (7, 8), on the other hand, made use of the ceric sulfate-arsenious acid colorimetric reaction, but introduced a dry ashing procedure in place of the wet ashing and distillation employed by Chaney. Lein (9) also adopted the ceric sulfate-arsenious acid reaction in the determination of blood inorganic iodine.

Salter and McKay (7) have discussed the factors that influence the rate at which iodide catalyzes the reduction of ceric ions by arsenious acid. Since the reaction is sensitive to the presence of extraneous materials (especially mercury and osmium), it would seem, from a theoretical standpoint at least, that the dry ashing procedure introduced by Salter and McKay would not be as satisfactory as a wet ashing procedure combined with a

distillation, as described by Chaney. A distillation would be expected to free iodine more effectively from non-volatile contaminants that interfere in the colorimetric reaction.

Salter and McKay report that, "The surface of the colorimeter tubes should be kept equilibrated with the reaction medium until just before use. Otherwise apparent loss of iodine will occur." This difficulty was not encountered in this laboratory. Thorough cleaning of the colorimeter tubes with chromic acid *immediately before use* was found sufficient for good results.

Chaney (3) reported that he had encountered a batch of phosphorous acid with which he obtained poor recoveries of iodine. In such cases he found that the recoveries could be improved if hydrogen peroxide was added after the phosphorous acid in the distillation. It was found here, however, that the addition of hydrogen peroxide was frequently followed by the appearance of a slight green color in the distillate, apparently indicating the presence of chromium. This color was not present when hydrogen peroxide was omitted. Since the recoveries of radioactive iodine were not decreased by the omission of the peroxide, its use was discontinued.

We are indebted to Dr. A. L. Chaney of the Los Angeles County Hospital for valuable advice rendered during the course of this work.

SUMMARY

1. The Chaney method for the determination of plasma iodine has been tested with the aid of radioactive iodine (I^{131}). Data on its reliability are presented. · The method was found to be satisfactory for the determination of the total or the protein-bound iodine contained in 3 cc. of plasma.

2. Values for total and protein-bound iodine of plasma of several species are reported. The levels of protein-bound iodine in the plasma of dog, rat, mouse, and domestic fowl were found to be quite similar (3 to 4 γ per cent). The amounts contained in human plasma were appreciably higher (5 to 8 γ per cent).

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THE DETERMINATION OF THYROXINE IN THE THYROID GLAND OF THE RAT*

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Most procedures used for the separation of thyroxine from other iodine-containing compounds of the thyroid gland are based on either the method of Harington and Randall (1) or that of Leland and Foster (2). In the former the thyroxine is separated from diiodotyrosine by precipitation in a slightly acid medium, whereas in Leland and Foster's method thyroxine is extracted with butyl alcohol from an alkaline hydrolysate of thyroid tissue. Leland and Foster observed that their method yielded much lower values for the thyroxine content of thyroid material than did the method of Harington and Randall; they attributed this to the presence of diiodotyrosine in the acid-insoluble fraction.

Blau (3) modified the method of Leland and Foster by adjusting the alkaline hydrolysate to pH 3.5 to 4.0 before its extraction with butyl alcohol. The thyroxine values obtained by this modification are 10 to 20 per cent higher than those obtained by the original Leland and Foster method, but still much lower than those obtained by the acid precipitation procedure of Harington and Randall (4).

The methods referred to above were designed for the analysis of relatively large amounts of thyroid tissue. Foster (5) subsequently adapted the butyl alcohol extraction procedure for the determination of the thyroxine content of the guinea pig thyroid. In the present communication a modification of Foster's method is described. Data on the thyroxine content of the rat thyroid are also presented.

EXPERIMENTAL

Hydrolysis of Thyroid Tissue—Rats weighing 200 to 300 gm. were anesthetized with nembutal and thoroughly bled. The thyroid glands were then rapidly excised, weighed, and transferred to 15 cc. graduated centrifuge tubes. 1 cc. of 2 N NaOH was added to each tube and the hydrolysis carried out in a steam bath. The tubes were placed in a rack made to fit into the hole of a steam bath; the lower half of each tube was in direct contact with steam. During the first 10 minutes the tubes were occasionally shaken until solution of the tissue occurred. They were then covered with glass

bubble stoppers and the hydrolysis allowed to proceed for about 15 hours. The flow of steam was adjusted to permit only slight evaporation of liquid.

Butyl Alcohol Extraction—The hydrolysates were neutralized by dropwise addition of 6 N H_2SO_4 and brought to pH 3.5 to 4.0, brom-phenol blue being used as an external indicator. 2 cc. of butyl alcohol were then added to each tube, the tubes tightly stoppered with rubber stoppers previously soaked in butyl alcohol, and their contents thoroughly shaken. The rubber stoppers were then removed, washed with 1 to 1.5 cc. of butyl alcohol, and the two phases in the tube separated by centrifugation. The butyl alcohol layer was withdrawn into another 15 cc. graduated centrifuge tube with the aid of a fine capillary tube and suction. The clear aqueous layer that remained was extracted a second time with 1.5 cc. of butyl alcohol. After centrifugation the second butyl alcohol extract was withdrawn into the tube containing the first extract. Thus both butyl alcohol extracts, the total volume of which did not exceed 6 to 7 cc., were now contained in a single centrifuge tube. An equal volume of 4 N NaOH containing 5 per cent Na_2CO_3 (3) was added to the butyl alcohol extracts, and the mixture thoroughly shaken. The two layers were separated by centrifugation. The upper butyl alcohol layer was drawn off into another 15 cc. centrifuge tube, extracted again with 5 cc. of the 4 N NaOH-5 per cent Na_2CO_3 solution, and two layers separated by centrifugation. The butyl alcohol layer, which now contains only thyroxine, was drawn off with suction into the two-neck digestion flask for the determination of its iodine content. The three aqueous phases derived from each sample were combined for the determination of non-thyroxine iodine.

Determination of Iodine—The butyl alcohol in the two-neck flasks was removed under reduced pressure in a water bath at 50 to 60° in a stream of CO_2. By this procedure this extract was brought to dryness. It was found essential to remove completely the butyl alcohol, since only a small amount of residual alcohol will reduce the chromic acid used in the subsequent digestion procedure. To the residue were added 25 cc. of 70 per cent H_2SO_4 and then 2 cc. of 60 per cent CrO_3 solution. It was observed that unless an appreciable amount of organic material was present during the digestion, the recovery of iodine was frequently very low. Hence before digestion of both thyroxine and non-thyroxine fractions, approximately 100 mg. of iodine-free wheat were added to each sample. Without any other modifications than the above, the digestion and distillation of the samples were carried out exactly as described for plasma in the preceding paper (6). The distillates were usually made up to a volume of 25 or 50 cc., and 1 or 2 cc. aliquots used for the colorimetric determination. Equal aliquots from a blank distillate were added to each of the standard samples used in the preparation of the standard curve.

For the determination of the iodine content of the non-thyroxine fraction, a 2 or 3 cc. aliquot was usually taken from the combined aqueous phases. This was added to a mixture of 100 mg. of iodine-free wheat, 25 cc. of 70 per cent H_2SO_4, and 2 cc. of chromic acid in the two-neck digestion flask. Digestion, distillation, and colorimetric determination were carried out exactly as described above for thyroxine iodine.

Test of Method

Recovery of Added Thyroxine and Diiodotyrosine—1 gm. of dried defatted rat muscle was hydrolyzed with 80 cc. of 2 N NaOH on a steam bath for 15

TABLE I

Separation of Thyroxine and Diiodotyrosine Added to Rat Muscle Hydrolysate

Thyroxine iodine added*	Diiodotyrosine iodine added†	Iodine recovered in thyroxine fraction		Iodine recovered in diiodotyrosine fraction	
γ	γ	γ	*per cent*	γ	*per cent*
5.65	12.2	4.50	80	11.5	94
5.65	12.2	5.05	89	10.0	82
5.65	12.2	5.35	95	10.9	89
5.65	12.2	4.69	82	10.4	85
3.26	6.28	2.60	80	5.20	83
3.26	6.28	2.85	87	6.25	100
3.26	0	2.60	80		
0	6.25			6.05	96
4.73	11.3	4.35	92	10.2	90
4.73	11.3	4.15	88	10.7	95
4.73	11.3	4.15	88	10.4	92
4.73	11.3	4.15	88	10.5	93
4.73	0	4.00	85	0.3	
4.73	0	3.80	80	0.3	
0	11.3	0.1		9.7	86
0	11.3	0.2		9.6	85

* Crystalline thyroxine from E. R. Squibb and Sons was used. Its iodine content was taken as 65.3 per cent.

† Diiodotyrosine dihydrate from the Amino Acid Manufactures, Chemistry Department, University of California at Los Angeles was used. Its iodine content was taken as 54.2 per cent.

hours. The mixture was then filtered. Known amounts of thyroxine and diiodotyrosine were added to the filtrate, and 1 cc. aliquots of the mixture subjected to the separation procedure described above. The results of several experiments are recorded in Table I.

When both thyroxine and diiodotyrosine were added to muscle hydrolysate, the recoveries of thyroxine iodine amounted to 87 per cent (average).

Slightly lower recoveries were observed when only thyroxine was added. These findings suggest that the separation procedure does not prevent a small fraction of the diiodotyrosine from appearing in the thyroxine fraction.

The low recoveries shown in Table I must be due in part to the method used for the iodine determination, which, as noted in the preceding paper, yields values that are about 5 per cent low. Even if the procedure for separation described here were free of error, the recoveries of added thyroxine and diiodotyrosine would probably not have been higher than 95 per cent. Since the recoveries of thyroxine found here amounted to 87 per cent (average), a loss of 5 to 10 per cent is probably encountered in the separation procedure.

Loss of Thyroxine during Alkaline Hydrolysis—Leland and Foster (2) observed that only 83 per cent of the thyroxine added to a sample of thyroid

TABLE II

Breakdown of Added Thyroxine during Hydrolysis of Desiccated Thyroid with 2 N NaOH

Sample No.	Desiccated thyroid hydrolyzed	Thyroxine iodine initially present	Thyroxine iodine added*	Thyroxine iodine found	Recovery of added thyroxine iodine	
	mg.	γ	γ	γ	γ	*per cent*
1	10.8	5.1	5.0	8.8	3.7	74
2	11.3	5.4	5.0	9.1	3.7	74
3	13.4	6.3	5.0	10.0	3.7	74
4	11.6	5.5	5.0	9.2	3.7	74

* Crystalline thyroxine from E. R. Squibb and Sons was used. Its iodine content was taken as 65.3 per cent.

tissue before hydrolysis with 2 N NaOH can be recovered by extraction with butyl alcohol. This suggests that hydrolysis of the thyroid protein with 2 N NaOH destroys an appreciable fraction of the liberated thyroxine. Other evidence obtained by Leland and Foster, however (2), showed that the amount of thyroxine iodine obtained after hydrolysis of desiccated thyroid with 2 N NaOH did not decrease appreciably between 16 and 50 hours of hydrolysis. These workers therefore postulated that destruction of the thyroxine built into the thyroid protein is not nearly so great as the destruction of added thyroxine.

Results similar to those of Leland and Foster were obtained by the method of hydrolysis and separation used here. The recovery of crystalline thyroxine that had been added to desiccated thyroid before treatment with 2 N NaOH amounted to 74 per cent (Table II). This value should be compared with the average recovery of standard thyroxine samples that

were subjected to the separation procedure but not to treatment with 2 N NaOH. The latter amounted to 87 per cent (Table I). These findings suggest that approximately 15 per cent of added thyroxine is destroyed

TABLE III

Effect of Duration of Hydrolysis on Thyroxine Determination in Desiccated Thyroid

Sample No.	Weight of sample	Duration of hydrolysis with 2 N NaOH	Thyroxine iodine found	
	mg.	*hrs.*	γ	*per cent of desiccated thyroid*
1	11.6	7	5.45	0.047
2	12.7	7	5.10	0.040
3	9.2	7	4.75	0.052
4	10.3	14	4.90	0.048
5	9.4	14	4.40	0.047
6	11.7	14	5.65	0.048
7	12.8	14	5.50	0.043
8	9.9	20	4.80	0.048
9	12.4	20	5.75	0.046
10	11.4	20	5.40	0.047

TABLE IV

Thyroxine Iodine and Total Iodine Content of Rat Thyroids

Weight of rat*	Weight of thyroid glands	Total iodine in thyroid glands	Thyroxine iodine in thyroid glands		
			Total	Per cent of wet weight of gland $\times 10^3$	Per cent of total iodine
gm.	*mg.*	γ	γ		
280	27	6.2	1.8	6.7	29
254	29	7.4	2.0	6.9	27
260	27	5.6	1.4	5.2	25
282	21	7.1	2.1	10.0	30
210	17	4.1	1.1	6.5	27
222	30	7.0	2.0	6.7	29
255	17	5.3	1.4	8.2	26
260	20	5.2	1.3	6.5	25
210	15	5.1	1.4	9.3	27
235	20	5.1	1.4	7.0	27
247	27	7.3	1.7	6.3	23

* The diet fed was composed of 68.5 per cent wheat, 5 per cent casein, 10 per cent fish meal, 10 per cent alfalfa, 1.5 per cent sodium chloride, and 5 per cent fish oil.

during the hydrolysis with 2 N NaOH. However, when samples of desiccated thyroid were hydrolyzed for periods varying from 7 to 20 hours, the amount of thyroxine found did not decrease as the time of hydrolysis in-

creased (Table III). This may mean, as Leland and Foster have suggested, that the thyroxine released from thyroid protein during treatment with 2 N NaOH does not react to the alkali in exactly the same manner as *added* thyroxine.

The work of Blau (4) indicates that the use of 8 per cent $Ba(OH)_2 \cdot 8H_2O$ instead of 2 N NaOH for hydrolysis yields higher thyroxine values when butyl alcohol is employed for separation. He ascribes the higher values obtained with $Ba(OH)_2$ to a diminished destruction of thyroxine. Blau's results were confirmed here, but the thyroxine values obtained with barium hydroxide were more variable than those with 2 N NaOH. Hence, 2 N NaOH was retained as the hydrolytic agent.

Analysis of Rat Thyroids—The thyroxine and non-thyroxine iodine of thyroid glands of rats fed a diet that contained 0.3 γ of iodine per gm. was measured by the separation procedure described above. The results recorded in Table IV show that this procedure yields values for thyroxine that are quite consistent. Thus in eleven rats the thyroxine content varied from 0.0052 to 0.010 per cent of the wet weight of the tissue. The mean value was 0.0072 per cent and the standard deviation ± 0.0013 per cent. Thyroxine iodine as a percentage of total iodine of the gland varied from 23 to 30.

<div align="center">SUMMARY</div>

A procedure suitable for the determination of 1 to 2 γ of thyroxine in thyroid tissue is described.

Data on the thyroxine content of the rat thyroid are presented.

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METHIONINE DETERMINATION IN PROTEINS AND FOODS*

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Our interest in the physiological relationship that exists between methionine and cystine in intermediary metabolism led us to seek reliable methods for the determination of these amino acids. Colorimetric methods, if they are specific, offer certain advantages in regard to speed and accuracy, especially if a spectrophotometer is used for estimating the color intensities. In a previous communication (1) we presented a modification of Sullivan's colorimetric method for cystine and its adaptation to the spectrophotometric procedure.

McCarthy and Sullivan (2) in 1941 proposed a colorimetric method for the determination of methionine in proteins, based on the red color formed when an alkaline solution of methionine and sodium nitroprusside is acidified. They noted, however, that tryptophane and histidine both produced a red color under similar conditions. They destroyed the tryptophane by acid hydrolysis and stated that the addition of glycine would eliminate the color produced by histidine. The experimental evidence offered in the present paper justifies our modification of the McCarthy-Sullivan method and indicates lower values for the methionine content of proteins than those found by investigators who have used the McCarthy-Sullivan procedure.

EXPERIMENTAL AND DISCUSSION

A Coleman spectrophotometer[1] was used to establish spectral transmission curves for the red color formed in the McCarthy-Sullivan test for methionine (Fig. 1). These curves show that 510 mμ is the wave-length of minimum transmission. The solutions from which Curve 1 was obtained had an excess of methionine, whereas the solutions from which Curves 2, 3, and 4 were obtained had an excess of sodium nitroprusside. It is observed from the relative position of these curves that the excess sodium nitroprusside absorbed light and this absorption somewhat changed the shape of the curves. However, in no case was the minimum transmission

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* This is the eighth in a series of papers on amino acids in staple foods.

[1] All measurements of color intensities reported in this paper were made with a Coleman spectrophotometer, model 10-S, with a monochromator slit of 7.5 mμ width.

shifted from the 510 mμ wave-length. Glycine was not added to the test solutions used in these experiments and the spectrophotometric readings were made against a blank[2] of distilled water.

After the wave-length of minimum transmission was determined, a calibration curve was established over a range of 0.25 to 1.5 mg. of methionine with 20 mg. of sodium nitroprusside and 10 mg. of glycine with the other reagents necessary for color development (Fig. 2, Curve 2). This is the procedure of McCarthy and Sullivan (2), except that the quantity of
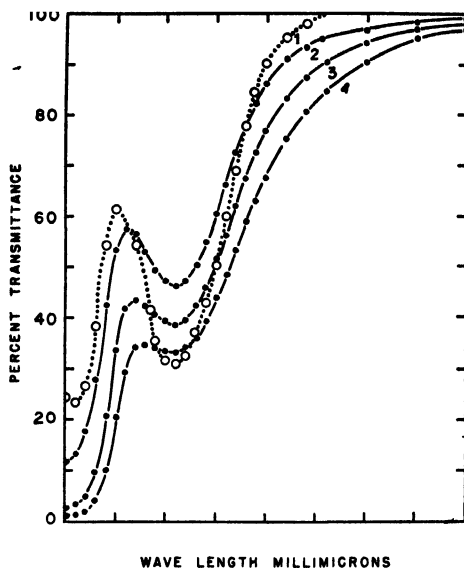
FIG. 1. Spectral transmission curves for the red color formed in the McCarthy-Sullivan test for methionine. Curve 1, 10 mg. of methionine and 2 mg. of sodium nitroprusside in the reaction mixture; Curve 2, 0.5 mg. of methionine and 10 mg. of sodium nitroprusside in the reaction mixture, Curve 3, 0.5 mg. of methionine and 20 mg. of sodium nitroprusside in the reaction mixture; Curve 4, 0.5 mg. of methionine and 30 mg. of sodium nitroprusside in the reaction mixture.

sodium nitroprusside and the concentration of the acid and alkali recommended by Hess and Sullivan (3) were used. Hereinafter this will be referred to as the McCarthy-Sullivan-Hess procedure. The calibration curve developed by this procedure did not show a straight line relationship

[2] Throughout this work all spectrophotometric readings were made with blanks of distilled water rather than with such blanks as McCarthy and Sullivan (2) recommended. Neither of these blanks is perfect, because the same error may be introduced into the determination by either of them; namely, the possible effect on the development of the methionine color caused by the other amino acids which are present in all protein hydrolysates.

between per cent transmittance and the quantity of methionine present.

As sodium nitroprusside is the color-developing reagent, it is important to have a suitable quantity of this reagent in the test solutions. McCarthy and Sullivan (2) used 30 mg. of this reagent, which was later changed by Hess and Sullivan (3) to 20 mg. No reasons were given by the authors for selecting these quantities or why 20 mg. were preferable to 30 mg. In some
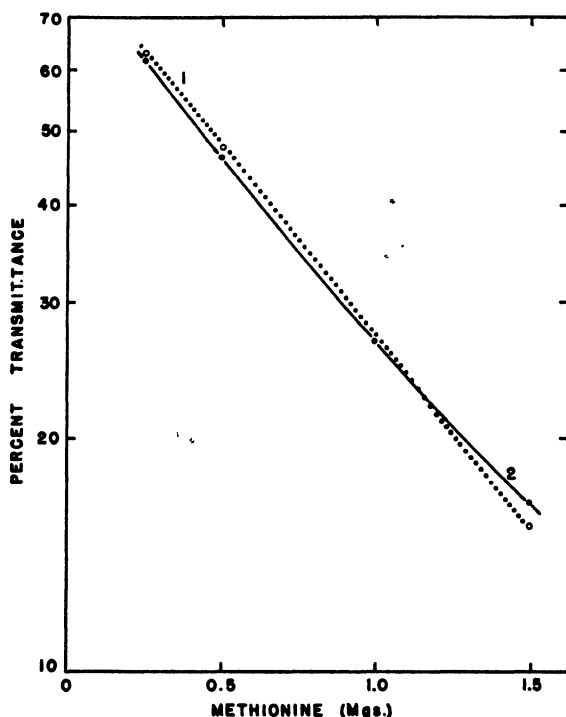
Fig. 2. Spectral transmission curves showing the effect of the addition of glycine to a blank. Curve 1, 20 mg. of sodium nitroprusside in the reaction mixture; Curve 2, 20 mg. of sodium nitroprusside and 10 mg. of glycine in the reaction mixture.

of our preliminary experiments, in which different quantities of sodium nitroprusside were added to solutions that contained an excess of methionine, but no glycine, a stoichiometric relationship between the reagent and methionine was indicated (4). The results in Table I show that a relationship of approximately 2:1 exists. We found that the stoichiometric relationship of 2:1 is no longer valid when glycine is added to the reaction mixtures (see Table II). This finding prompted us to investigate in detail the rôle of glycine in the McCarthy-Sullivan reaction.

The transmission curves in Fig. 3 show the effect of the addition of glycine

to a blank that contained 20 mg. of sodium nitroprusside. The blank that contained no glycine shows at 510 mμ a transmission of 65 per cent, whereas the one that contains glycine has a considerably higher transmission of 84 per cent.
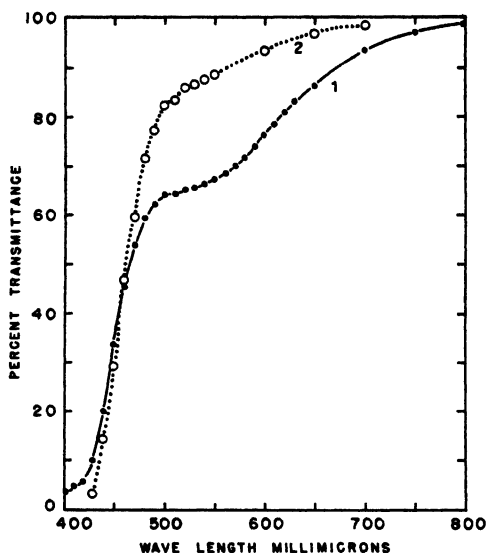
FIG. 3. Spectral transmission curves showing the effect of the addition of glycine to the reaction mixture containing sodium nitroprusside. Curve 1, 20 mg. of sodium nitroprusside in the reaction mixture; Curve 2, 20 mg. of sodium nitroprusside and 10 mg. of glycine in the reaction mixture.

TABLE I

Experiments in Which Excess Methionine (10 Mg.) Was Used to Demonstrate Stoichiometric Relationship between Sodium Nitroprusside and Methionine

Sodium nitroprusside	Transmission	Methionine that reacted with sodium nitroprusside*	Ratio of sodium nitroprusside to methionine that reacted
mg.	*per cent*	*mg.*	
2	28.1	0.97	2:0.97
3	16.0	1.47	2:0.98

* The methionine values were obtained from calibration Curve 1, Fig. 2.

The addition of glycine to the reaction mixtures which contained methionine likewise resulted in a considerable increase in transmission. For example, a transmission of 22 per cent was obtained for 1 mg. of methionine with 20 mg. of sodium nitroprusside and a transmission of 27 per cent with 10 mg. of glycine added. This increase in the transmission at first was thought to be caused only by the reaction between the glycine and the excess sodium nitroprusside. However, further investigation revealed

that glycine also decreased the red color produced by the methionine-nitroprusside reaction. Comparison of Experiments 1 and 2 (Table II) shows that the addition of glycine caused a 74 per cent loss in the methionine value. In these experiments the methionine and sodium nitroprusside were present in the reaction mixture in stoïchiometric quantities. However, the addition of glycine caused only a small change in transmission when an excess of either methionine or sodium nitroprusside was present in the

TABLE II

Effect of Glycine on Methionine-Nitroprusside Reaction

Experiment No.	Quantities used			Transmission	Methionine found*	Differences observed in found methionine values	
	Sodium nitroprusside	Methionine	Glycine				
	mg.	*mg.*	*mg.*	*per cent*	*mg.*	*mg.*	*per cent*
1	3	1.5	0.0	18.0	1.36	0.0	0.0
2	3	1.5	10	56.5	0.35	−1.01	−74.0
3	20	1.5	10	16.5	1.44	+0.08	+5.8
4	3	10.0	10	18.2	1.35	−0.01	−0.7
5	10	1.5	10	19.0	1.31	−0.05	−3.7

* The methionine values were obtained from calibration Curve 1, Fig. 2. In Experiment 4 the quantity of nitroprusside in the reaction mixture is a limiting factor for the "found methionine" value.

TABLE III

Errors Caused by Addition of Histidine to Test Solutions Containing Methionine
McCarthy-Sullivan-Hess procedure used.

Added histidine	0.5 mg. methionine	1 mg. methionine
mg.	*per cent*	*per cent*
0.4	+10	+14
0.8	+30	+23
1.0	+43	+29

reaction mixtures (Experiments 3, 4, and 5). The common ion effect is a plausible explanation of this phenomenon and a slight excess of sodium nitroprusside in the reaction mixture is apparently an advantage.

The results in Table III indicate that glycine is not effective in discharging all of the histidine-nitroprusside color when there is as little as 0.4 mg. of histidine present in the reaction mixture. It is also shown that when the quantity of methionine in the reaction mixture is constant the percentage error increases as the quantity of histidine is increased. Accordingly, because of the error caused by the presence of histidine, there is reason to believe that the actual methionine values of protein hydrolysates are lower than those obtained with the McCarthy-Sullivan-Hess procedure.

It is evident that histidine, the added glycine, and the other amino acids in the reaction mixture are the factors that influence the methionine-nitroprusside reaction. Therefore, the most desirable approach to the problem would be to separate methionine from the ·other constituents of protein hydrolysates. Toennies and Kolb (5) were able to precipitate methionine quantitatively with mercuric salts from a solution that contained no other amino acids. We found that, when HgCl₂ was added to a hydrolysate of not more than 40 mg. of casein, methionine was precipitated quantitatively at pH 7.5, but some of the other amino acids, including histidine, were also precipitated. Thus, precipitation with mercuric salts offered no advantage.

The next attempt was to remove histidine from the hydrolysate. Phosphotungstic acid, the well known precipitant for the basic amino acids, was used. The experimental conditions under which the phosphotungstic acid precipitation was carried out permitted the complete removal of histidine from the hydrolysate without the precipitation of any of the methionine. This finding made it possible to perform the nitroprusside test for methionine without the addition of glycine.

The elimination of glycine should restore the stoichiometric relationship that has been shown to exist between nitroprusside and methionine, but some amino acids other than methionine in the test solution react with nitroprusside and invalidate this relationship. Our results show that no specific quantity of nitroprusside is optimum for different protein hydrolysates and widely different aliquots of the same hydrolysate. It appears that the requirement for this reagent is to a great extent proportional to the amino acids other than methionine that are left in the test solution. We found that when the test solution was limited to an aliquot of the hydrolysate that represented not more than 50 mg. of protein material the use of 10 mg. of nitroprusside gave the most consistent methionine values.

The proposed modification of the McCarthy-Sullivan (2) method is as follows:

The protein material is hydrolyzed in boiling 20 per cent HCl for 24 hours. The hydrolysate is cooled and transferred quantitatively to a volumetric flask with distilled water. The flask should be of such volume that the diluted solution will have an HCl concentration of about 4 to 5 per cent. This concentration was found to be suitable for precipitation of the basic amino acids with phosphotungstic acid. An aqueous solution of phosphotungstic acid (1 gm. per ml.) is added dropwise until precipitation is complete[3] and then a few drops are added in excess. The solution is then made to volume with distilled water. It is allowed to stand in an ice water

[3] Centrifugation at frequent intervals during the addition of the phosphotungstic acid is necessary in order to determine when the precipitation is complete. The volumetric flask is used as the container for centrifugation.

bath for 2 hours, after which it is centrifuged and again placed in the bath for $\frac{1}{2}$ hour before the supernatant liquid is filtered. After the filtrate has reached room temperature, an aliquot containing not more than 300 mg. of the original protein material is transferred to a 25 ml. volumetric flask. NaOH (5 N) is added dropwise until the solution turns light blue and is still acid to litmus paper (pH 4 to 5). The solution is then made to volume with distilled H_2O, decolorized with 50 mg. of carboraffin, and filtered. An aliquot of 4 ml. or less of the filtrate is transferred to a large test-tube. To this 2 ml. of NaOH (5 N) are added and a sufficient quantity of distilled water to bring the volume to 6 ml. Then 1 ml. of a freshly prepared 1 per cent sodium nitroprusside solution[4] is added and the tube placed in a water bath for 8 minutes at 40°. The tube is then placed in an ice water

TABLE IV

Methionine Values Obtained with McCarthy-Sullivan-Hess Procedure and Proposed Modification

Material	Aliquot	McCarthy-Sullivan-Hess procedure		Proposed modification	
		Aliquot	Material	Aliquot	Material
	ml.	*mg*	*mg.*	*mg.*	*mg.*
Casein (1 gm., air-dry)	1	0.56	28.0	0.315	24.6
	2	1.10	27.4	0.65	25.4
	3	1.63	27 1	0.95	24.8
One chicken egg	1	0.495	272.0	0.315	216
	2	0.960	264.0	0.628	216
	3	1.405	258 0	0.910	209

bath for 5 minutes, after which 2.5 ml. of 20 per cent HCl are added slowly while the tube is being shaken vigorously. The shaking is continued in the bath until the characteristic red color develops (about 1 minute) and then shaken for 2 minutes at room temperature. Within 4 to 5 minutes after the addition of the HCl the per cent transmission is determined and the corresponding methionine value is read from the calibration Curve 1, Fig. 2, which is prepared according to the procedure described above. If the solution becomes slightly turbid after the addition of the HCl, it should be centrifuged before the per cent transmission is determined.

The data shown in Table IV present a comparison of the results of the estimation of the methionine content of casein and a whole chicken egg by the McCarthy-Sullivan-Hess procedure and the proposed modification. The methionine values obtained by the McCarthy-Sullivan-Hess procedure are higher than those obtained by the proposed modification. Further-

[4] It is essential that exactly 10 mg. of sodium nitroprusside be present in the reaction mixture, because this is the quantity used in making the calibration curve.

more, they are not consistent when aliquots of different size are used.   It
may be observed that the methionine values for the chicken egg are lower

TABLE V

Composition of Protein Preparations

Proteins	Moisture	Ash	Nitrogen, on moisture- and ash-free material
	per cent	*per cent*	*per cent*
Casein	7.03	0.25	16.07
Gliadin (wheat)	7.06	0.60	17.37
α-Glutelin (wheat)	9.35	0.21	17.14
Zein	4.69	0.12	15.99
Glycinin "55"	7.56	0.97	17.11
Egg albumin	6.05	0.2	15.98

TABLE VI

Methionine Content of Some Proteins and Foods

Material	Material represented in aliquot	Trans-mission	Methionine found*		Average	Methionine content calculated to moisture- and ash-free basis
			Aliquot	Material		
	mg.	*per cent*	*mg.*	*per cent*	*per cent*	*per cent*
Casein	12.80	59.0	0.310	2.42		
	25.60	40.2	0.650	2.54		
	38.40	28.5	0.950	2.47	2.48	2.72
Gliadin (wheat) .	25.60	55.5	0.325	1.46		
	51.20	37.2	0.720	1.40	1.43	1.54
α-Glutelin (wheat)	29.80	48.0	0.500	1.68		
	44.70	36.6	0.735	1.64	1.66	1.81
Zein	25.60	48.0	0.500	1.95		
	51.20	27.6	0.950	1.85	1.90	2.0
Glycinin "55"	22.85	62.6	0.260	1.14		
	45.70	45.0	0.525	1.15	1.14	1.25
Whole wheat flour	124.40	63.2	0.250	0.20		
	186.60	55.5	0.375	0.20		
	248.80	50.5	0.450	0.18	0.2	0.22†
Soy bean meal (defatted)	25.60	72.0	0.140	0.55		
	51.20	61.5	0.280	0.55		
	76.80	53.0	0.410	0.53	0.55	0.61†
Egg albumin (crystalline)	10.00	57.3	0.345	3.45		
	20.00	37.6	0.710	3.55	3.50	3.73

* The methionine values were obtained from calibration Curve 1, Fig. 2.
† The methionine content was calculated on a moisture-free basis.

as the size of the aliquot is increased; yet it is significant that the lowest
value is still considerably higher than those obtained by the proposed
modification.

In order to demonstrate the applicability of the proposed method for methionine, samples of diversified types of proteins and foods were selected. The casein sample was prepared according to'the method of Van Slyke and Baker (6). The preparation of α-glutelin of wheat has been described in a previous article (7). The glycinin "55" was prepared from defatted Illini soy bean meal by a method previously described (8). Table V gives data on the protein preparations used to establish the degree of their purity.

Our findings are collected in Table VI. It should be pointed out that the quantities of material given in the second column are the quantities present in the aliquots of the hydrolysates before precipitation with phosphotungstic acid and, therefore, are larger than those actually present in the reaction mixtures. Methionine values for the different size aliquots are in good agreement. This is also true for duplicate hydrolysates of the same protein. As a typical example, hydrolysates of two samples of our zein preparation gave methionine values of 1.96 and 2.0 per cent.

The values reported here are lower than those obtained by McCarthy and Sullivan (2) and Hess and Sullivan (3). However, they are in reasonable agreement with those reported by Lavine (9) and Albanese, Frankston, and Irby (10).

The authors wish to express their thanks to Miss Marguerite A. Sabol, who rendered valuable technical assistance.

<div align="center">SUMMARY</div>

1. It was found that 510 mμ was the wave-length of minimum transmission for the red color formed in the methionine-nitroprusside reaction of McCarthy and Sullivan.

2. Data are presented which indicate a stoichiometric relationship of approximately 2:1 between sodium nitroprusside and methionine. The presence of other amino acids, particularly glycine, invalidates this relationship.

3. A modification of the McCarthy-Sullivan method for the estimation of methionine in protein and food material is presented and its adaptation to the spectrophotometric procedure is described.

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LETTERS TO THE EDITORS

PARTIAL SEPARATION OF ADENOSINETRIPHOSPHATASE FROM MYOSIN

Sirs:

According to Engelhardt and Ljubimowa,[1] myosin, representing about 40 per cent of the muscle protein, is identical with ATPase.[2] This view is accepted by nearly all investigators in this field owing to their inability to effect any kind of separation. According to Szent-Györgyi,[3] repeated recrystallizations of myosin do not alter the enzymatic activity.

Expressing the activity as Q_p = c.mm. of H_3PO_4 split at 38° per hour by 1 mg. of protein (22.4 c.mm. = 31 γ of P), we usually obtain with 5 minutes incubation of purified myosin from rabbit muscle with optimal concentrations of ATP (3×10^{-3} M), borate-KCl (0.015 M), and Ca^{++} (1.5 $\times$ 10^{-3} M), at pH 9.2, Q_p values between 1500 and 2500, which are increased about 20 per cent by addition of 0.01 M KCN.[4] Such myosin solutions, either of reprecipitated myosin prepared according to Edsall or redissolved crystallized myosin of Szent-Györgyi, can be divided by means of a lanthanum salt precipitation into fractions of higher and lower enzymatic activity.   With about 0.5 cc. of 0.1 M La(NO₃)₃ for 40 mg. of myosin in 20 cc. of 0.5 M KCl and 0.03 M NaHCO₃, nearly all the protein is pre-

Example No.	Fraction	P split per 5 min.	Protein	Q_p
		γ	γ	
1	Original myosin (crystalline)	57	198	2,500
	Eluate from La ppt.	22	33	5,800
2	Original myosin (Edsall)	25	90	2,400
a	Eluate	25	54.5	4,000
b	" centrifuged	22	42	4,500
3	Original myosin (Edsall)	53	82	5,600
a	Residual solution after pptn.	18	28	5,600
b	Eluate from La ppt.	39	22	15,400

P determined[5] as P split from ATP present in 1.7 cc. of incubation mixture; protein, by micro-Kjeldahl determination.

[1] Engelhardt, W. A., and Ljubimowa, M. N., *Nature*, **144**, 668 (1939).
[2] ATPase = adenosinetriphosphatase; ATP = adenosine triphosphate.
[3] Szent-Györgyi, A., *Acta physiol. scand.*, **9**, suppl. 25 (1945).
[4] Binkley, F., Ward, S. M., and Hoagland, C. L., *J. Biol. Chem.*, **155**, 681 (1945).
[5] Lohmann, K., and Jendrassik, L., *Biochem. Z.*, **178**, 419 (1926).

cipitated with the La salt. After washing with a KCl-NaHCO$_3$ solution, several fractions can be eluted by means of a KCl-NaHCO$_3$ buffer solution containing KCN (0.01 M) and ATP (1 × 10^{-3} M).

The first and the second eluates show an activity 2 or 3 times that of the original solution, especially if all traces of the La salt are removed. The Q_p is then in the range from 4000 to 7000 and in certain cases, when the original myosin is very active, even higher (Example 3).

Although we have not been able to increase the activity by reprecipitation of the active fractions with La salts, nevertheless, we conclude that the ATPase is contained only in a fraction of the myosin. Slight physicochemical differences enable this fraction to be separated partially from the bulk of the myosin.

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THE CHEMICAL NATURE OF FLAVACIDIN

Sirs:

In 1943 McKee and MacPhillamy[1] and Bush and Goth[2] reported almost simultaneously on the production by *Aspergillus flavus*, grown in submerged culture, of an antibiotic factor which closely resembled penicillin in its biological and chemical properties. The antibiotic behavior of this agent was further studied by McKee, Rake, and Houck[3] who adopted for it the name "flavacidin." Recently Bush, Goth, and Dickison[4] described extraction and chromatographic procedures for the purification of this substance, designated by them "flavacin." The most potent preparation which they secured by these methods assayed 800 Oxford units per mg.

The purpose of the present communication is to report that in April, 1944, we isolated the penicillin produced by *Aspergillus flavus* in the form of the crystalline sodium salt. Its potency is 1400 units per mg. against *Staph. aureus*, and the *B. subtilis-Staph. aureus* ratio is 0.72 (*B. subtilis* strain 558-K). The analytical data were not entirely conclusive, but ·suggested the empirical formula $C_{14}H_{20}O_4N_2S$ for the free acid. This is also the composition of penicillin F,[5] the penicillin elaborated by certain strains of *Penicillium notatum*. Degradation with mercuric chloride yielded an aldehyde $C_8H_{13}O_2N$ which was isolated in the form of the 2,4-dinitrophenylhydrazone. However, from comparison of the melting points and x-ray diffraction patterns[6] it appears that this derivative is not identical but isomeric with the 2,4-dinitrophenylhydrazone of 3-hexenoylaminoacetaldehyde, which is the corresponding degradation product of penicillin F.[5] There is evidence that the aldehyde derived from flavacidin is a double bond isomer of the latter, namely 4-hexenoyl-aminoacetaldehyde, so that the variable group R in the general penicillin formula $C_9H_{11}O_4N_2S \cdot R$[5] would be represented in this penicillin by $-CH_2 \cdot CH_2 \cdot CH{=}CH \cdot CH_3$.

[1] McKee, C. M., and MacPhillamy, H. B., *Proc. Soc. Exp. Biol. and Med.*, **53**, 247 (1943).

[2] Bush, M. T., and Goth, A., *J. Pharmacol. and Exp. Therap.*, **78**, 164 (1943).

[3] McKee, C. M., Rake, G. W., and Houck, C. L., *J. Bact.*, **47**, 187 (1944).

[4] Bush, M. T., Goth, A., and Dickison, H. H., *J. Pharmacol. and Exp. Therap.*, **84**, 262 (1945).

[5] Committee on Medical Research, Office of Scientific Research and Development, Washington, and the Medical Research Council, London, *Science*, **102**, 627 (1945).

[6] We wish to express our sincere thanks to Dr. R. D. Coghill and Dr. N. C. Schieltz of the Northern Regional Research Laboratory, United States Department of Agriculture, Peoria, Illinois, for performing and interpreting the x-ray diffraction measurements carried out in connection with this study.

It should be also mentioned that ultraviolet absorption measurements on the crystalline sodium salt revealed the presence of small amounts of penicillin G (R = —$CH_2 \cdot C_6H_5$).

The details of this investigation will be made available in the projected publication of all the experimental material contributed by the groups which during the war have collaborated in the chemical study of penicillin organized by the Office of Scientific Research and Development.

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THE EFFECT OF TRYPTOPHANE ON THE SYNTHESIS OF NICOTINIC ACID IN THE RAT*

Sirs:

In 1942 we demonstrated that the feeding of casein, of individual amino acids, and of other nitrogenous compounds led to synthesis of nicotinic acid in rats kept on a protein-free diet.[1]   In view of the recent findings of Krehl *et al.*[2] of the ability of tryptophane to replace nicotinic acid as a growth factor in rats maintained on a corn diet, we investigated the effect of tryptophane on the excretion of nicotinic acid derivatives in the rat.[3]   These were measured in the urine of five adult male rats (175 to 225 gm.) fed a diet containing purified casein (or gelatin) 15, sucrose 78, corn oil 3, salt mixture 4, choline 0.2, cystine 0.15 per cent, plus adequate amounts of the B vitamins, except nicotinic acid.   The averaged results are given in the table.

Average Excretion of Nicotinic Acid Derivatives per Rat per 24 Hours

Diet	Days	NA*	N¹-Me†
		γ	γ
15% casein	1–10	35	90
15% gelatin	11–18	15	38
15% " + 50 mg. *l*-tryptophane, orally.	19–22	347	985
15% "	23–26	43	43
15% " + 50 mg. *dl*-tryptophane, subcutaneously	27–28	175	580
15% casein	29–34	28	35
15% " + 100 mg. *dl*-tryptophane, orally	35–36	325	770

* NA includes all nicotinic acid derivatives hydrolyzable with 6 N HCl by the method used by Huff and Perlzweig [1]

† N¹-Methylnicotinamide as determined by the method of Huff, Perlzweig, and Tilden [5]

The data show a significant drop in the nicotinic acid excretion when casein was replaced by gelatin, a tryptophane-deficient protein.   The in-

[1] Huff, J. W., and Perlzweig, W. A., *J. Biol. Chem.*, **142**, 401 (1942).

[2] Krehl, W. A., Teply, L. J., Sarma, P. S , and Elvehjem, C. A., *Science*, **101**, 489 (1945).

[3] While these studies were in progress, Singal *et al.* published an abstract (Singal, S. A., Briggs, A. P., Sydenstricker, V. P., and Littlejohn, J., *Federation Proc.*, **5**, 154 (1946)) of their work stating that rats on a diet of corn grits excreted increased amounts of unmethylated nicotinic acid upon the addition of tryptophane.   In our experiments the major increase is in the methylated form.

crease in the nicotinic acid excretion on addition of 50 mg. of *l*-tryptophane, when given either orally or parenterally, was immediate, within 24 hours, and much greater than those observed previously with other amino acids.[1] The addition of tryptophane to the casein diet likewise produced a large rise in nicotinic acid excretion. Indeed the increase in nicotinic acid synthesis was apparently so great that it exceeded the capacity of the rat to methylate it, and much of it was excreted as such in the urine. This fraction was shown to consist entirely of free nicotinic acid by failure to obtain larger values after hydrolysis with 6 N HCl, and its identity was definitely confirmed by assays with *L. arabinosus*. The identity of the methylated fraction as N^1-methylnicotinamide was established by obtaining practically identical values from analyses by three methods based on different chemical reactions.[4-6] The fecal excretion of nicotinic acid derivatives did not vary significantly after the administration of tryptophane, amounting to about 12 γ per rat per day. These observations indicate that tryptophane may be the important precursor of nicotinic acid synthesis in the rat.

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Sirs:

In a recent note[1] experiments were described which showed that immunological reactions occurred between a film of antigen deposited on a metal slide and homologous antibodies, in spite of an intervening screen of one or more layers of barium stearate. That work has been extended by substituting screens of Formvar (a formaldehyde polyvinyl polymer) for barium stearate without any significant difference in results.

The analogy between enzymatic and immunological reactions naturally led to testing the possibility that enzymes could react on a deposited film of protein notwithstanding an intervening screen of inert material. Tests were carried out with the crystalline proteolytic enzymes pepsin and trypsin. For this study, films of the antigen bovine albumin were deposited on

Trypsin Action on Bovine Albumin through Formvar Screens

	Antigenic films, 1 double layer egg albumin plus					
	1 double layer bovine albumin		2 double layers bovine albumin		3 double layers bovine albumin	
	A	*A*	*A*	*A*	*A*	*A*
Thickness of Formvar screen........	0–10	65	0–50	80	0–65	150
" " adsorbed antibovine antibodies after trypsin treatment and removal of screen..............	0	85	0	100	0	130

polished metal slides, as described in the previous note. Trypsin solution, for instance, when smeared on the slide acted upon the films in a few seconds so that subsequent treatment of the plate by homologous antisera did not bring about any increase in thickness, thus demonstrating the inactivation of the films by the enzyme. If, however, a drop of solution of Formvar in ethylene dichloride was deposited on the transferred antigen layers, a protecting screen was formed on the protein film. After formation of the Formvar screen a drop of trypsin solution (0.4 per cent in veronal, pH 7.5) was deposited on the screen for 5 minutes and the plate then washed. The Formvar film was subsequently dissolved in ethylene dichloride and finally the plates were treated with homologous antisera to test the antigenic films for their ability to react with antibodies. It was ascertained that antigenic films deposited on metal slides and coated with Formvar retained their ability to react with antibodies after removal of the Formvar screen.

My experiments have shown that: (1) for a given number of deposited

[1] Rothen, A., *Science*, **102**, 446 (1945).

antigenic films there is a corresponding critical thickness of Formvar film above which the screens offer complete protection against the enzymatic action; (2) the greater the number of deposited antigenic films originally present the larger the critical thickness of the screen necessary for protection.

It is shown in the table that a screen 65 A thick prevents the inactivation of one underlying double layer of bovine albumin, whereas the same screen offers no protection if there are three, and very little protection if there are two, underlying double layers.   Similar results were obtained when screens of barium stearate were substituted for the Formvar.   If diffusion of the enzyme took place, it would be hard to understand why a screen 65 A thick protected one double layer of antigen and permitted the complete inactivation of three underlying double layers of the same antigen. .

These experiments suggest the possibility that proteolytic enzymes may operate at distances somewhat greater than 100 A.   If confirmed by work on other similar systems, it would mean that no direct contact is necessary between enzyme molecules and the molecules subjected to disintegration. Enzymatic action may thus originate through a field of forces resulting from the extended resonators suggested by London.   A system of many layers of proteins could in this way be more unstable towards the action of the proteolytic enzyme than a system made of fewer layers of the same protein.

The biological implications of such a hypothesis are obviously numerous and will be discussed in full elsewhere together with the work now actively in progress.   .

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THE EFFECT OF THE SULFOXIDE FROM *dl*-METHIONINE ON GLUTAMIC ACID AND GLUTAMINE METABOLISM*

Sirs:

It has been shown recently[1] that the sulfoxide from *dl*-methionine is a growth-inhibiting analogue of glutamic acid in *Lactobacillus arabinosus* with an antibacterial index of 75 (total inhibition). The inhibition is overcome specifically by glutamic acid but not by glycine, alanine, methionine, aspartic acid, or asparagine. The corresponding sulfone does not inhibit bacterial growth in equivalent concentrations.

If glutamic acid is replaced by glutamine in the synthetic medium,[2] even twice the concentration of the sulfoxide which inhibits bacterial growth completely in the presence of glutamic acid has little influence on the growth of the bacteria. The findings, shown in the table, suggest that methionine

l(+)-Glutamic acid	*l*(+)-Glutamine	Sulfoxide	Bacterial growth; optical density
mg. per ml.	*mg. per ml.*	*mg. per ml.*	
0	0	0	0.03
0.12	0	0	0.48
0.12	0	8	0.05
0.48	0	8	0.46
0	0.12	0	0.48
0	0.12	8	0.48
0	0.12	10	0.47
0	0.12	16	0.43

sulfoxide prevents the amidation of glutamic acid to glutamine. The sulfoxide may therefore serve as a useful tool in the study of glutamic acid metabolism.

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[1] Borek, E., Sheiness, P., and Waelsch, H., *Federation Proc.*, **5**, 123 (1946).
[2] Hac, L. R., Snell, E. E., and Williams, R. J., *J. Biol. Chem.*, **159**, 273 (1945).

FOLIC ACID AND PEROSIS*

Sirs:

In studies on the requirement of the chick for folic acid, perosis was found to occur on a purified diet which was adequate in choline, biotin, and manganese, but contained only 50 mg. of inositol per 100 gm. instead of the usual 100 mg. Perosis in chicks receiving ample amounts of manganese and all of the B vitamins known at the time was reported as early as 1942. Richardson, Hogan, and Miller[1] observed that at least four factors were

EFFECT OF FOLIC ACID ON PEROSIS IN CHICKS
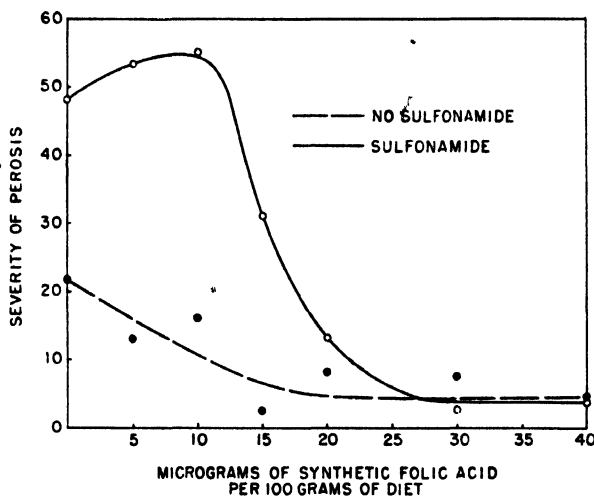
necessary to prevent perosis in chicks. These were manganese, choline, biotin, and an "unidentified organic nutrient present in the eluate of a fuller's earth adsorbate of a water extract of beef liver." McGinnis, Norris, and Heuser,[2] using a diet containing adequate manganese, found that yeast contains a perosis-preventing factor (or factors) that is different from choline and biotin. Campbell, McCabe, Brown, and Emmett[3] observed

[1] Richardson, L. R., Hogan, A. G., and Miller, O. N., *Univ. Missouri, Res. Bull.* *343* (1942).

[2] McGinnis, J., Norris, L. C., and Heuser, G. F., *Poultry Sc.*, **21**, 474 (1942).

[3] Campbell, C. J., McCabe, M. M., Brown, R. A., and Emmett, A. D., *Am. J. Physiol.*, **144**, 348 (1945).

perosis in chicks that were receiving high levels of vitamin B_c (folic acid), but not in those receiving low levels.

Contrary to this, we have found that perosis varies inversely with the amount of folic acid in the diet. Succinylsulfathiazole added to the diet at the 2 per cent level accentuated the perosis, but had no unfavorable effect upon the growth and hemoglobin formation. The perosis was corrected by adding synthetic folic acid[4] to the diet.

Fifteen day-old white Leghorn chicks per lot were used in this experiment. The degree of perosis was first determined at 4 weeks of age. Afterwards the perosis became so severe that the chicks were unable to secure food and water, and many died before the end of the 6 week experimental period. The perosis score was determined according to the formula proposed by Wilgus, Norris, and Heuser.[5]

The data are summarized in the accompanying graph. On the sulfonamide diet the low levels of folic acid caused an increase in perosis, due probably to the greater weight of the chicks. Under the conditions of the experiment the data indicate that on the non-sulfonamide diet approximately 20 γ of folic acid per 100 gm. of diet were necessary to prevent perosis, whereas on the sulfonamide diet about 30 γ were needed. The fact that the severity of perosis was greater on the sulfonamide diet and that more folic acid was needed to overcome the perosis suggests that folic acid is not concerned directly, but is acting indirectly by stimulating the intestinal microorganisms to produce the antiperotic factor.

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[4] We are indebted to the Lederle Laboratories, Inc., Pearl River, New York, for the synthetic folic acid.

[5] Wilgus, H. S., Jr., Norris, L. C., and Heuser, G. F., *Poultry Sc.*, **16**, 232 (1937).

STUDIES ON THE METABOLISM OF BRAIN SUSPENSIONS

III. RESPIRATION AT LOW OXYGEN TENSION*

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Most studies of tissue respiration have been carried out in air, that is at a partial pressure of oxygen of 155 mm. of Hg, allowing for water vapor pressure at 38°, or in 95 or 100 per cent oxygen. The partial pressure of oxygen to which tissues *in vivo* are subjected is considerably lower than these values. The arterial and venous oxygen tensions are only about 80 and 40 mm. of Hg and the actual value in the neighborhood of respiring tissue cells is presumably much lower. Davies[1] found that the oxygen tension at the surface of cat brain cortex varies from the full arterial tension down to at least as low as 5 mm. of Hg. It is therefore necessary to know whether the respiration rates as ordinarily measured are likely to be the same as in the presence of physiologically occurring oxygen tensions.

In the use of tissue slices in oxygen it is recognized that the oxygen tension at various depths within the slice will vary, but it is assumed that even at a very low oxygen tension the rate of respiration is the same as at higher tensions. The original calculation of Warburg (1) regarding the permissible thickness of a tissue slice is based on this assumption, although at the time the calculation was made the only evidence supporting the assumption was provided by work on microorganisms. Nearly all workers have accepted the limitations to the thickness of slices as calculated by Warburg's equation rather uncritically. Fuhrman and Field (2), however, have shown with liver slices that there is a distinct optimum thickness, 0.48 to 0.62 mm., at which maximum rate of respiration in an atmosphere of oxygen occurs. Slices thinner than the optimum respire more slowly, probably because the proportion of damaged cells is greater; slices thicker than the optimum respire more slowly presumably because an adequate oxygen tension is not maintained in the inner layers of the slice. The optimum thickness for liver slices as found by Fuhrman and Field agreed well with the calculated maximum thickness and provides indirect evidence that liver cells respire at their full rate at very low oxygen tensions.

¹ Davies, P. W., personal communication.

The results of studies on the effect of oxygen tension on respiration rates of tissue slices (3, 4) probably reflect only a mixture of aerobic metabolism of superficial cells, anaerobic metabolism of inner layers, and possible intermediate conditions. Warren (4) has made the first direct and valid study of the effect of very low oxygen tension on an animal tissue. He used suspensions of bone marrow and followed the rate of change of oxygen tension in the medium by a polarimetric method, and was thus able to show that oxygen was utilized by the tissue at a constant rate until the oxygen tension was reduced to 4 mm. of Hg or less. Winzler (5) has shown by the polarographic method that the oxygen tension at which the respiration rate of yeast begins to fall off varies widely with the temperature, being 0.6, 2.5, and 6 mm. of Hg at 5°, 20°, and 34° respectively.

With tissue suspensions the particles are small enough to allow the assumption that all parts of the tissue are subjected to the same oxygen tension, provided the suspension is adequately mixed. As was pointed out by Dixon and Elliott (6), the rate of oxygen consumption in manometric experiments can be limited by the rate of diffusion of oxygen from the gas phase into the fluid rather than by the activity of the respiring system, if the rate of shaking is too low or if the rate of oxygen absorption by the system is too high. (The effect of increasing the rate of shaking is not alone a reliable indicator of adequate oxygen diffusion (Winzler (5).) With low oxygen tensions in the atmosphere only low rates of respiration can be measured without fear of limitation by diffusion. It was shown previously (7) that the rate of respiration (per unit weight) of brain suspensions prepared in isotonic medium is not affected by the concentration of the suspension and is of the same order as the rate of respiration of slices. By using dilute brain suspensions, with low absolute rates of oxygen uptake, it is therefore possible to obtain reasonably valid direct information concerning the effects of oxygen concentration on the rate of respiration of brain tissue.

The results so far obtained show that the rate of respiration of brain tissue is unaffected by decreasing the oxygen tension in the gas phase to as low as about 4 mm.[2] of Hg. The low oxygen tensions found by Davies on the brain surface *in vivo* are thus sufficiently high to maintain the maximum rate of respiration, but if circulation were decreased, the rapid respiration would quickly reduce the oxygen tension below the critical level. The calculation concerning permissible thickness of slices in oxygen is evidently reasonably valid as far as maintenance of an adequate oxygen concentration in the inner layers of the slice is concerned. On the other hand, it was previously shown (7) that an atmosphere of 100 per cent oxygen has a

[2] In the abstract (*Federation Proc.*) this figure was incorrectly given as 0.4 mm. of Hg.

slowly developing inhibitory effect on the respiration of brain suspensions or slices. The toxic effect of high oxygen tension should be borne in mind in work with slices and oxygen.

In the course of this work a convenient method for determining low concentrations of oxygen in gas mixtures was developed and is described below.

Methods

Gas mixtures of air and commercial nitrogen, or of mixtures of oxygen and nitrogen containing 5 per cent CO_2, were prepared and stored over saturated calcium chloride solution in a system of large aspirator bottles. After thorough mixing the oxygen content was determined, usually by the method described below.

Suspensions of whole rat brain were prepared by homogenization in warm Ringer-0.033 M phosphate-0.01 M glucose medium (calcium omitted). After homogenization the pH of the suspension was readjusted to about 7.3 with drops of 0.1 N sodium hydroxide. Suitable quantities of suspension and medium, total 3 cc., were pipetted into Barcroft manometer flasks equipped with bored stoppers in the side arms. The center tube contained splayed rolls of alkali-soaked paper held clear by small glass collars (7). The gas to be used was passed in series through the experimental vessels (usually four) at room temperature at about 400 cc. per minute for 5 minutes. (Connections were made so that the pressure in the left and right (experimental) vessels was equalized so that the manometer fluid was not blown over.) After the gas was passed in, all taps were closed and the flasks were placed in the bath at 38°, at half minute intervals for convenience in reading, and shaking started. The manometer taps were opened briefly immediately and then occasionally during the 8 minute equilibration period to release the pressure developed by warming. Thereafter readings were taken at suitable intervals.

Method for Determining Low Oxygen Concentrations—For determining low concentrations of oxygen the following method was found to give accurate results. Both main vessels of a Barcroft differential manometer received 2.5 cc. of N NaOH. The side bulb of the left vessel received 0.8 cc. of M NaCl in 0.002 N H_2SO_4; the side bulb of the right vessel received 0.8 cc. of a solution containing 0.1 M pyrogallol in M NaCl-0.002 N H_2SO_4. The gas to be analyzed was passed through the flasks in series (right first), at a rate of about 400 cc. per minute for 5 minutes, at room temperature. The taps were then closed, the vessels were placed in the bath at 38°, shaking started, and the taps quickly opened and closed immediately and several times later, as long as appreciable pressure due to warming remained. (The pressure could be detected by opening one tap at a time.) When the manometer reading became constant, a zero reading was taken

and the contents of the bulbs were tipped into the vessels. The alkaline pyrogallol then absorbed the oxygen in the right vessel, and when the reading became constant, after about 30 minutes, it was noted. The percentage of oxygen in a mixture of oxygen and nitrogen was then given by the following equation:

$$\% \text{ oxygen} = \frac{100hk}{\left(V_g \dfrac{273}{311} \times \dfrac{710}{760}\right)} = \frac{100hk}{0.82V_g} = 122\,\frac{hk}{V_g}$$

where h is the change in reading, k is the oxygen constant for the right vessel, V_g is the volume of gas space in the right vessel, and corrections are applied for temperature and the vapor pressure of water at $38°$.

The method can be used satisfactorily also with gas mixtures containing 5 per cent CO_2. Although the alkali absorbs CO_2, ample alkalinity remains, after the gas is passed in, to cause the pyrogallol to absorb oxygen, and the expansion due to warming at $38°$ is more than sufficient to give a positive pressure after complete absorption of the CO_2, so that no air is drawn in when taps are briefly opened if time is allowed for the vessels to warm up first. When 5 per cent CO_2 is present, the above equation becomes

$$\% \text{ oxygen} = \frac{100hk}{0.82V_g \times \dfrac{100}{95}} = 116\,\frac{hk}{V_g}$$

The use of M NaCl as solvent for the pyrogallol is necessitated by the fact that addition of water or a solution of appreciably different osmolarity from that of the NaOH solution in the main vessel causes a large change in vapor pressure which is difficult to balance out with the control vessel. Considerably more dilute alkali and pyrogallol are used than is commonly used for oxygen absorption, since otherwise it is difficult to avoid changes in vapor pressure. There was no evidence that CO was evolved in appreciable amount during oxygen absorption. The vapor pressure effect of the 0.1 M pyrogallol is small enough to be negligible. On tipping the pyrogallol solution in from the bulb there is an immediate slight increase in pressure. This is evidently a heat effect, and tests with nitrogen purified by hot copper and by alkaline pyrogallol showed that this pressure disappears rapidly.

The highest concentration of oxygen which can be measured by this method depends upon the density of the manometer fluid used. With kerosene it is about 2 per cent. With Clerici's solution, density 4, the limit is about 8 per cent. The figures in Table I give results of duplicate determinations and a comparison with the results of determinations by the method of Van Slyke (8).

The method could be modified for use with the Warburg apparatus. 'It would, of course, be useful only when a rather large volume of the gas to be analyzed is available, but it is easier than standard methods and more suitable for low oxygen concentrations. For the present work the method had the advantage that a manometer for gas analysis could be set up in series with experimental manometers and the oxygen content of the gas determined at the same time as the respiration.

Results

Results of a typical experiment showing the effects of lowered oxygen tension are illustrated in Fig. 1. In air the respiration rate per unit weight of tissue is unaffected by varying the concentration of tissue or by increasing the rate of shaking. In 4.3 per cent oxygen the rate is the same as in air and unaffected by varying low tissue concentration, but as the concentration of tissue is raised above 50 mg. per cc., diffusion effects enter so

TABLE 1

Percentage of Oxygen Found in Various Gas Mixtures

Method	CO₂-free gases			5 per cent CO₂		
Van Slyke		0.81	2.44	0.49	1.93	4.58
		0 79	2.51	0.61	1.85	4 56
Proposed	Passed over hot copper	0 58	2 43	0 52	1.92	4.59
	0.00 0.00	0 62	2 40	0.44	1.90	4 52

that the rate per unit weight is lowered and can be increased by increasing the rate of shaking.

As lower oxygen tensions were tried, the critical tissue concentration was lowered, and with the low oxygen uptake rates occurring in highly diluted suspensions it was naturally difficult to rule out small extraneous effects. But when curves through numerous observed points were smoothed, duplicates agreed well and results were reproducible. However, as shown in Figs. 2 and 4, one effect of extreme dilution was not eliminated. On decreasing the amount of tissue below 8 mg. per cc., the apparent rate per unit weight was often initially a little low and increased after about 30 minutes. This effect was probably an artifact connected with the slow rate at which equilibrium is reached between CO₂ evolution by the tissue and absorption by the alkali paper.

Fig. 2 shows the results of an experiment in which vessels were filled with commercial nitrogen containing 0.6 per cent oxygen. This gave the lowest oxygen tension tested, 4 mm. of Hg. It will be seen that, at least after the first 30 minutes, the rates per unit weight were about the same at all

tissue concentrations in air and at the two lowest concentrations in "nitrogen." At higher tissue concentrations in "nitrogen" the rate was limited by diffusion.

There was no obvious change in the respiratory quotient under conditions in which the respiration rate was definitely limited by lack of oxygen. R.Q. values of 1.0, 0.93, 0.85, and 0.91, determined in bicarbonate-buffered medium with the Dixon-Keilin apparatus, were obtained with 50 mg. of tissue per cc. in 0.6, 0.6, 0.9, and 1.9 per cent oxygen. These figures are approximate since the total respiration was low, but they show no con-
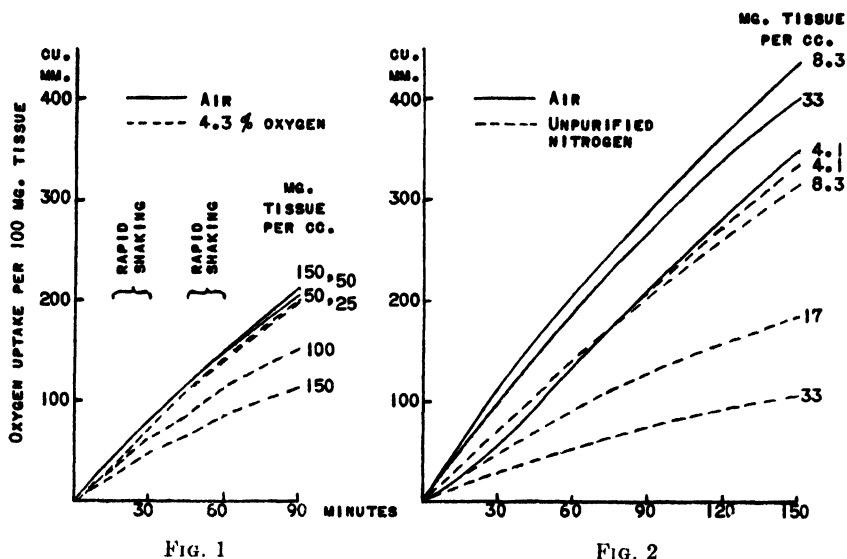
Fig. 1.

Fig. 2.

FIG. 1. Effects of tissue concentration and rate of shaking on respiration in different concentrations of oxygen. Normal shaking rate, 120 oscillations per minute; rapid shaking, 150 oscillations per minute.

FIG. 2. Effects of tissue concentration on respiration. Unpurified nitrogen means gas direct from the nitrogen cylinder containing 0.6 per cent of oxygen.

sistent difference from values obtained with suspensions or slices in the presence of ample oxygen (9, 10). Laser (11) found the R.Q. of retina and chorion to be lowered by low oxygen tension while the oxygen uptake rate was still unchanged. With bone marrow, Warren (4) found no change in R.Q. even when the oxygen uptake rate was decreased by 50 per cent.

When the tissue was respiring at its full rate the pH of the medium showed little change, but whenever the rate was limited by lack of oxygen in the medium the pH fell appreciably. The pH after 120 minutes in the experiments of Fig. 2, for instance, was 7.1 to 7.3 in all manometers in which the full rate occurred, but with 50 and 100 mg. of tissue in 0.6 per cent oxygen

it was 7.05 and 6.8. Evidently glycolysis sets in as soon as the oxygen concentration in the medium is insufficient to support full respiration. Fig. 3 shows the results of an experiment in which the suspending medium was Ringer-bicarbonate, the gases contained 5 per cent CO_2, and alkali papers were absent during the experimental period. Dixon-Keilin apparatus was used so that the total oxygen uptake could be measured simultaneously. In this experiment movement of the manometer fluid during
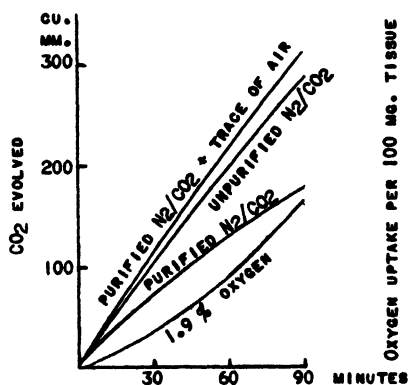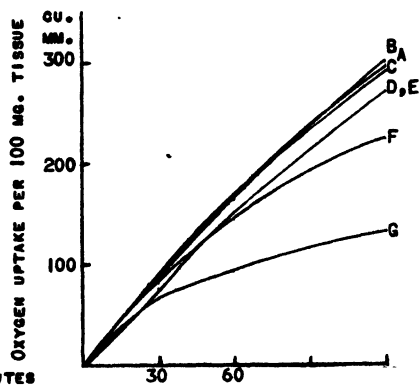
FIG. 3.   FIG. 4.

FIG. 3. Glycolysis in bicarbonate-buffered medium in the presence and absence of small amounts of oxygen. 150 mg. of tissue in 3 cc. The unpurified N_2/CO_2 contained 0.5 per cent of oxygen. Purified N_2/CO_2 is gas completely freed of oxygen by passage over hot copper oxide. The trace of air was introduced by withdrawing gas from the vessels by means of a syringe and rubber valve connected to the manometer, until the manometer reading indicated that 870 c.mm. had been removed, and then allowing air to replace the gas removed. This gave approximately 0.5 per cent oxygen in the vessel.

FIG. 4. Effects of glucose concentration and tissue concentration on respiration in air, with examples of duplicate determinations. Curve A, 33 mg. of tissue per cc., 0.005 M glucose; Curve B, 8.3 mg. per cc., 0.005 M glucose; Curve C, 8.3 mg. per cc., 0.0005 M glucose; Curves D and E, duplicates, 4.1 mg. per cc., 0.005 M glucose; Curve F, 8.3 mg. per cc., 0.0002 M glucose; Curve G, 8.3 mg. per cc., no added glucose.

the experimental period registered CO_2 evolution by glycolysis, since oxygen uptake and respiratory CO_2 evolution approximately balanced each other. The amount of tissue present was too high to allow full respiration at 0.5 per cent oxygen (unpurified nitrogen-CO_2) and was at about the critical concentration for the 1.9 per cent oxygen. It will be seen that the glycolysis rate in the presence of a little oxygen is higher and better maintained than in complete anaerobiosis (Elliott and Henry, Paper IV (12)). In 1.9 per cent oxygen there was practically no glycolysis at first, but, as the oxygen tension was reduced by respiration, the rate steadily increased until at 90 minutes it was nearly as high as in the presence of 0.5 per cent

oxygen.   The oxygen uptakes over the 90 minutes were 85 and 170 c.mm. in the 0.5 and 1.9 per cent (initial) oxygen respectively.   This absorption of oxygen would have reduced the initial 1.9 per cent oxygen to about 1.2 per cent.   While this method shows changes in glycolysis rate, it does not show changes in respiration rate with time.   But the results seem to indicate that glycolysis by brain tissue sets in as the respiration is decreased by lowering oxygen tension.   This is in agreement with results of Craig and Beecher (3) on brain cortex.   But since these authors worked with sliced tissue, no conclusion can be drawn from their results as to the actual oxygen tension at the cells at which these effects occur.   Warren (4) found similar effects with slices and suspensions of bone marrow.   With various types of red and white blood cells, Kempner (13) found that glycolysis did not increase until a marked decrease in respiration had occurred.   With various other tissues, glycolysis has been found to increase before respiration is decreased by lowering the oxygen tension (Bumm *et al.* (14); Laser (11); Stern and Melnick (15); Craig and Beecher (16)).

In a previous paper (10) it was shown that the maximum respiration rate could be maintained with a glucose concentration of about 6×10^{-4} M. By taking advantage of the fact that respiration is not affected even by extreme dilution, it was possible to show that an even lower glucose concentration, 2×10^{-4} M, could maintain the full rate (see Fig. 4).   As the glucose concentration was further lowered as a result of consumption by the tissue, the respiration rate fell off.   Calculation, from the oxygen uptake, of the amount of glucose consumed during the equilibration and experimental periods shows that the glucose concentration at the time when the rate just began to fall below that of the controls (30 minutes), was about 1×10^{-4} M.   This is presumably the critical glucose concentration.   The limiting oxygen concentration, calculated from the solubility of oxygen at 4 mm. of Hg pressure, is about 5×10^{-6} M or less.

SUMMARY

The rate of respiration of brain tissue is unaffected by reduction of the oxygen tension as low as 4 mm. of Hg, provided that the rate of diffusion of oxygen from the gas phase into the suspension medium does not limit the rate of oxygen uptake.

The full rate of respiration can be obtained with a glucose concentration as low as 10^{-4} M.

A method for determining low concentrations of oxygen in gas mixtures is described.

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STUDIES ON THE METABOLISM OF BRAIN SUSPENSIONS

IV. ANAEROBIC GLYCOLYSIS*

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In previous papers of this series (1–3), studies on the aerobic metabolism of homogenized suspensions of whole brain were reported. It was shown that brain suspensions, prepared under proper conditions, respire at rates comparable with those of slices of gray matter and present a number of advantages in the study of brain tissue metabolism. Work with suspensions has facilitated the study of various factors which affect glycolysis, and these are here described.

Methods

Suspensions were prepared from whole rat brains by homogenization in warm medium, with the apparatus of Potter and Elvehjem (4). The Ringer-bicarbonate-glucose medium (RBG) used contained 0.12 M NaCl, 0.006 M KCl, 0.0012 M MgSO$_4$, 0.001 M phosphate, 0.024 M NaHCO$_3$, 0.007 M glucose, and, in some cases, 0.0025 M CaCl$_2$. The effects of the latter ion were variable but always small.

Glycolysis was followed manometrically in the Barcroft apparatus. The vessels contained a total of 3 cc. of fluid and were filled with 5 per cent CO$_2$ in nitrogen by the method previously described (3). Unless otherwise stated, the gas mixture was freed of oxygen by passage through an electrically heated tube filled with reduced copper fragments. In many experiments identically filled flasks were removed at zero time and at the end of the experimental period, samples were diluted and deproteinized with ZnSO$_4$ and NaOH, and lactic acid was determined on the filtrates by the method of Barker and Summerson (5). For pyruvate determinations, 1 cc. of 20 per cent trichloroacetic acid was run into the flask and the filtrate was analyzed by the method[1] of Bueding and Wortis (6).

[1] The color values for standard pyruvate solutions were very constant when Merck's reagent grade of ethyl acetate was used. Low values were obtained with samples of Baker's c.p. ethyl acetate, especially if it had been kept for some time. When the Baker product was treated overnight with anhydrous calcium chloride and distilled, it gave the same values as did the Merck product.

Results

Effect of Pyruvate Concentration—The effect of added pyruvate in stimulating glycolysis is well known (7, 8).   Fig. 1 shows that the initial rate is increased to the same extent by concentrations of added pyruvate between 0.05 and 2.0 mM per liter, but with the lower concentrations of pyruvate (or with higher concentrations of tissue) the rate quickly falls off, due to the destruction of pyruvate (see below).   A higher concentration, 5.0 mM, causes the rate to be lowered again appreciably.   The results shown in Fig. 2 were obtained by adding very small amounts of pyruvate and com-
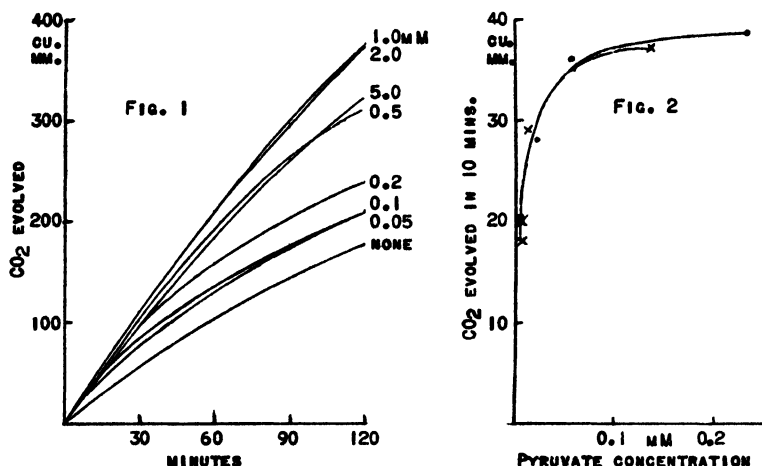
FIG. 1. Effect of pyruvate concentration on anaerobic glycolysis.   100 mg. of tissue. Bulbs, tipped in after 20 minutes equilibration, containing sufficient pyruvate to give the indicated millimolar concentrations of added pyruvate.

FIG. 2. 100 mg. of tissue.   Bulbs containing pyruvate to give added concentrations of 0, 0.05, 0.1, 0.3 mM, tipped in after 10 minutes equilibration.   ● represents CO_2 evolution between 5 and 15 minutes after addition of pyruvate; pyruvate determined in a separate identical sample at 10 minutes.   ×, CO_2 evolution between 25 and 35 minutes; pyruvate determined at 30 minutes

paring the rate over short periods with the pyruvate concentration analytically determined.   It will be seen that the maximum effect is obtained in the presence of about 0.05 mM pyruvate.   This concentration, 0.44 mg. per 100 cc., is of the same order or less than that normally present in blood.

Effects of Oxygen—Rosenthal (9) showed that the rate of glycolysis of slices of various tissues is increased, following a period of aerobiosis in the same medium.   This has been confirmed for brain suspensions, though the effect is rather short-lived, especially with thick suspensions.   The maximum effect is reached after about 10 minutes previous aerobiosis at

38°.   If the respirometer vessels are filled with nitrogen-CO_2 gas mixture as it comes from the cylinder, containing about 0.5 per cent oxygen, the rate of glycolysis is considerably and continuously greater than in the presence of purified gas mixture.[2]   Determinations of pyruvate have shown that these effects can be accounted for by increased amounts of pyruvate in the suspension (Table I).   Further, it was found that the rapid glycolysis occurring in the presence of optimal amounts of added pyruvate is not further increased by previous aerobiosis or by the presence of traces of oxygen (Fig. 3).

Rosenthal (9) found that glycolysis by liver slices could be stimulated by any suitable oxidizing agent, pyruvate, aldehyde, ferricyanide, or reducible dyes, and did not consider the effect of previous aerobiosis to be necessarily due to pyruvate formation.   Nevertheless there seems to be

TABLE I

Effects of Traces of Oxygen and of Washing on Pyruvate Concentration and Glycolysis

Tissue from 100 mg , fresh weight. of brain   Duplicate vessels were set up and pyruvate determined in one of each pair at 30 minutes

		Pyruvate concentration after 30 min	CO_2 evolution in 60 min.
		$M \times 10^6$	c mm
Unwashed tissue	No O_2	8	111
	Traces of O_2	22	164
Washed tissue	No O_2	24	169
	Traces of O_2	49	195

no doubt that pyruvate is accountable for these effects in brain.   It was previously shown (2) that traces of material estimated as pyruvate are always present in respiring brain suspensions.   Presumably an equilibrium concentration of pyruvate is reached when formation from glucose or lactate is balanced by oxidative destruction.   After a preliminary period of aerobiosis there is thus sufficient pyruvate present to stimulate glycolysis for a short time during subsequent anaerobiosis, but it is rapidly decomposed. In the presence of a trace of oxygen, some aerobic metabolism continues and maintains a sufficient concentration of pyruvate to stimulate glycolysis continuously.

Effect of Washing and of Tissue Extract—If the brain suspension is centrifuged and the sediment is washed once or twice, by resuspending in

[2] The effect is not due to inhibitory substances being produced in the process of freeing the gas from oxygen.   Glycolysis in vessels which were filled with purified gas and a measured small amount of air proceeded at the same rate as in unpurified gas.

cold RGB and recentrifuging, and then made up to the original volume in RBG, the rate of glycolysis is considerably greater than in the original suspension,[3] provided Mg ion is present (see below).   If the supernatant fluid obtained on centrifuging a strong fresh suspension is added to a washed suspension, the rate of glycolysis is depressed (Fig. 4).   The extract alone has slight glycolytic activity (Fig. 5).   The inhibitory factors are
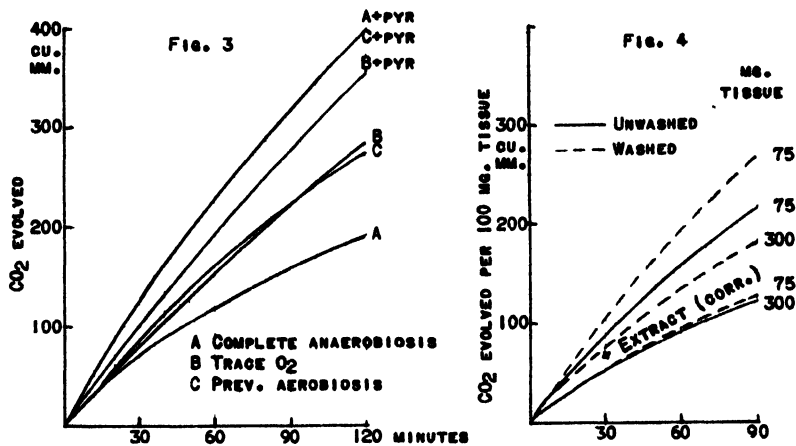
FIG. 3. Effects of previous aerobiosis and traces of oxygen in the absence and presence of added pyruvate.   75 mg. of tissue.   Curve A, in purified N_2-CO_2 gas; Curve B, in unpurified N_2-CO_2 (containing about 0.5 per cent O_2); Curve C, in purified gas following a period of 15 minutes shaking in O_2-CO_2 at 38°.   Pyruvate, where added, 0.002 M.

FIG. 4. Effects of washing, tissue concentration, and tissue extract.   The brain was homogenized 100 mg. per cc. in Ringer-bicarbonate-glucose medium.   Some of the suspension was centrifuged; the sediment was washed twice by making up with RBG to 5 times its original volume and recentrifuging, and then made up to its original volume.   The flasks received 0.75 cc. of original suspension, 0.75 cc. of washed suspension (each with 2.25 cc. of RBG), 0.75 cc. of washed suspension plus 2.25 cc. of extract (the first supernatant fluid), 2.25 cc. of extract plus 0.75 cc. of RBG, 3 cc. of original suspension, 3 cc. of washed suspension. CO_2 evolutions are given per 100 mg. of original tissue.   The curve for tissue plus extract is corrected for the activity of extract alone.

mostly removed in the first extraction and the supernatant fluid after several washings has no effect.   However, more of the inhibitor was found to be liberated from washed tissue during the course of experiments.

The increased glycolysis in washed suspensions can be accounted for by

[3] The fact that these supensions can be centrifuged and resuspended without loss of glycolytic activity (see also Geiger (10)) is another indication that homogenization in isotonic medium does not destroy all cell structure.   Cytolyzed cell preparations do not glycolyze rapidly without the addition of various factors concerned in the glycolytic mechanism (11, 12).

the presence of an increased concentration of pyruvate during glycolysis (Table 1). During the manipulations involved in setting up an experiment, the suspension is necessarily subjected to aerobic conditions for a time, and consequently a small amount of pyruvate is present when anaerobiosis

TABLE II

Destruction of Pyruvate by Extract

Pyruvate, M $\times$ 10^6, found after 30 minutes anaerobiosis at 38°.

100 mg. tissue, washed				No tissue	
No addition	Extract added	Pyruvate added	Pyruvate and extract	Pyruvate added	Pyruvate and extract
50	8	250	34	270	110

The extract was 2 cc. of the supernatant fluid obtained on centrifuging 150 mg. per cc. of fresh brain suspension.
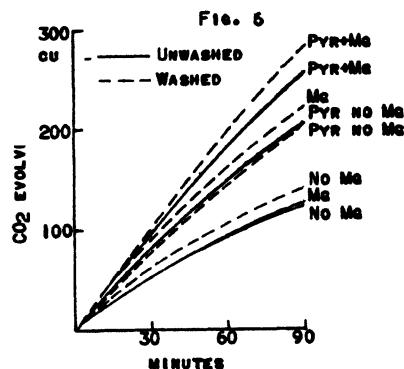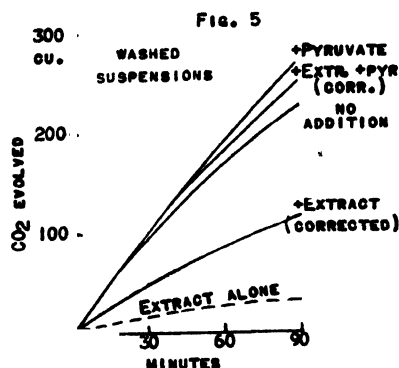
FIG. 5. Reversal of extract effect by pyruvate. Washed suspension from 100 mg. of fresh tissue. The extract used, 2 cc., was the supernatant fluid obtained on centrifuging a suspension of 150 mg. of fresh tissue per cc. Pyruvate, where added, 0.002 M. The curves for tissue plus extract have been corrected by deduction of the activity of the extract alone (which was not affected by added pyruvate).

FIG. 6. Effects of magnesium. The tissue was homogenized in Mg-free Ringer-bicarbonate-glucose and a portion was washed and resuspended in the same medium. Vessels received suspension from 100 mg. of fresh tissue. Magnesium added, 0.002 M $MgCl_2$; pyruvate, 0.001 M, added from side bulbs.

is established. The tissue extract contains factors which destroy pyruvate so that the initial concentration of pyruvate is more rapidly reduced in unwashed suspension, or in the presence of added extract, than in washed suspension alone (Table II). Traces of oxygen or the addition of pyruvate has little or no immediate effect with dilute washed suspensions (Figs. 5 and 6). The inhibitory effect of added extract on washed brain is largely abolished by added pyruvate (Fig. 5).

Extracts prepared from tissue homogenized with and without glucose have exactly the same effect. Most of their effect remains after heating to 100° for 15 minutes, centrifuging clear, and readjusting the pH by treatment with CO_2. The small amount of lactate in the extracts, 0.002 to 0.004 M, could not account for their effects. Chemical determination of lactate production proved that the effect was not due to CO_2 retention (see Table III, Experiment 1).

The respiration rate of these suspensions, measured in the Dixon-Keilin apparatus, is lowered about 22 per cent by washing. The addition of extract to washed suspensions increased the oxygen uptake by an amount about equal to the uptake of the extract alone.

Magnesium Ion—Geiger (11) showed that magnesium ion is necessary for the activity of glycolyzing extracts. The presence or absence of this ion in the medium usually has very little effect on ordinary isotonic suspensions, but the stimulating effect of added pyruvate is greater in its presence. The increase in glycolysis rate following washing does not occur if magnesium is omitted from the washing and suspension medium, although added pyruvate still increases the rate (Fig. 6). Presumably the tissue itself contains sufficient magnesium to make nearly full use of the very small traces of pyruvate ordinarily present in unwashed suspensions and to make suboptimal use of added pyruvate. Tissue washed with Mg-free medium contains more pyruvate, but insufficient magnesium to make maximum use of it. The maximum effect of magnesium is reached with about 0.002 M Mg and increasing the concentration to 0.005 M has little further effect.

Lactate—In the absence of added pyruvate, the addition of 0.01 M d-lactate inhibits the glycolysis rate appreciably, though the extent of inhibition is variable. (This concentration of lactate is considerably higher than that developed by glycolysis in most of these experiments.) In the presence of 0.001 M pyruvate, the addition of lactate had no effect (Fig. 8).

Effect of Tissue Concentration—The respiration rate, per unit weight of tissue, of brain suspensions prepared in isotonic medium is almost independent of the tissue concentration, though, with suspensions cytolyzed by suspension in hypotonic medium, the rate decreases with increasing dilution (1). As is shown in Fig. 7 and has previously been observed by Geiger (10), the rate of anaerobic glycolysis decreases markedly with increasing concentration. In unpurified gas the effects of traces of oxygen and tissue concentration produce a complex curve. At extreme dilution, the traces of oxygen are sufficient to maintain aerobic metabolism. With somewhat stronger supensions, diffusion of oxygen is inadequate to maintain respiration, so that glycolysis sets in at a high rate, due to the stimulation by traces of oxygen mentioned above. At still higher tissue concentrations,

the rate falls again, due to the inhibitory effect of increased tissue concentration on glycolysis.

The readily extractable factors which destroy pyruvate account for only a fraction of the inhibition by increased tissue concentration. With unwashed tissue, the same extremely low concentration of pyruvate, 5 to 9×10^{-3} mM, was found during complete anaerobiosis with thin as with thicker suspensions. There was a somewhat lower percentage inhibition by increased tissue concentration with washed tissue (Fig. 4), or with ample added pyruvate, but the effect was still marked. The higher lactate concentration produced in strong suspensions could not account for the inhibition (Fig. 8). Nor could the effect be due to change in pH, since halv-
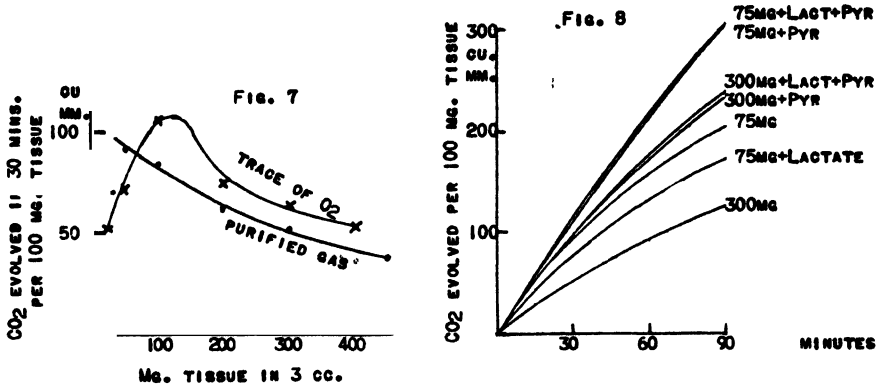
FIG. 7. Effect of tissue concentration in purified and unpurified N_2-CO_2 gas mixtures.

FIG. 8. Tissue concentration effect with optimal pyruvate and balanced lactate. The vessels contained 75 or 300 mg. of fresh tissue in 3 cc. Added pyruvate 0.002 M, added lactate 0.01 M. Lactate present at end (calculated from CO_2 evolution during experimental and preliminary periods): strong suspension plus pyruvate, 0.012 M; dilute suspension plus pyruvate and added lactate, 0.014 M.

ing the bicarbonate concentration in the medium scarcely affected the rate of glycolysis. Geiger (10) described an inhibitor of glycolysis in cytolyzed brain which was associated with cell débris, and Utter et al. (12) have identified this with a pyridine nucleotidase. Geiger reported that the inhibitor did not affect the glycolysis of uncytolyzed cells. Since washing does not lower the glycolysis of isotonic suspensions, pyridine nucleotides are presumably retained inside cell structures. It is difficult to ascribe the concentration effect in this case simply to coenzyme destruction. Possibly autolytic processes play a part.

Serum—The addition of rat serum or inactivated horse serum, 2 cc. in the total 3 cc., inhibited lactic acid formation (chemically determined)

about 35 per cent, when compared with a control containing calcium ion and as much added lactate as was present in the serum (approximately 0.009 mM per cc. of serum) (see Table III, Experiment 2). The serum was not found to destroy either initial traces or added pyruvate, and in itself contained sufficient material determined as pyruvate (about 0.1 mM) to cause stimulation. The nature of the inhibiting substance was not identi-

TABLE III

Effects of Phosphate Buffer and Other Factors on Total Acid and Lactic Acid Formation

Quantities are expressed in micromoles per gm of fresh tissue in 2 hours.

Experiment No	Medium	Tissue, mg per 3 cc	pH*		Acid† formed	Lactic acid formed
			Start	End		
1	Ringer-bicarbonate	75 (Washed)			143	125
	+ extract from 225 mg tissue	75 "			86	61
	Correction for extract alone				10	3
2	Ringer-bicarbonate	100			123	107
	+ d-lactate 0.008 M	100			107	99
	+2 cc horse serum(inactivated)	100				64
	+2 " rat serum	100				67
3	Ringer-bicarbonate	75			125	116
	"	300			61	51
	Ringer-0.033 M phosphate	75	7.37	7 08	176	84
	" " "	300	7 53	7 03	72	36
4	Ringer-0 032 M phosphate	100 (Washed)	7.22	6.91	154	104
	+ d-lactate 0 01 M	100 "	7 31	6 92	189	79
	+ extract from 300 mg. tissue	100 "	7.43	7.20	93	44

* pH determined with glass electrode. The initial pH was determined rapidly on the contents of an identically set up manometer flask, immediately after equilibration.

† The acid formed in bicarbonate medium is calculated from manometrically determined CO_2 evolution With phosphate medium the value for acid formation is the calculated amount of acid required to produce the observed change of pH in phosphate buffer of the concentration used

fied. Some inhibition occurred with the supernatant fluid from heated serum (pH readjusted with CO_2). Serum has no effect on, or slightly stimulates, the respiration of these suspensions. Human spinal fluid also exerted about 20 per cent inhibition of glycolysis.

Phosphate Buffer—The Ringer-phosphate-glucose medium (RPG) for these experiments usually contained 0.033 M sodium phosphate, pH 7.6, 0.102 M NaCl, and other constituents, as in RBG. The pH of suspensions

in RPG was about 7.0 immediately after homogenization and was adjusted to about 7.6 with drops of 0.1 N NaOH. Manometers were filled with CO_2-free nitrogen.

In RBG the total acid formation, as indicated by CO_2 evolution, was usually equivalent to or somewhat higher than the lactic acid production (Table III). In RPG the acid formation, as calculated from the change in pH of the buffered suspension on the basis of the buffering capacity of the phosphate alone, was greater than in RBG, while the lactic acid production was considerably lower than in RBG. The acid formation in RPG was very much higher than the amount calculated from the lactic acid production. The nature of the extra acid has not been determined. Preliminary experiments in the absence of any buffer suggest that the peculiar anaerobic metabolism in RPG may be due to lack of bicarbonate or CO_2. This point is being studied. It may be relevant to note that Geiger (10) found that the lactic acid formation in RPG could be brought to the same level as in RBG by the addition of adenosine triphosphate. The inhibitory effects of increased tissue concentration and tissue extract were similar in both media (Table III, Experiments 3 and 4).

. *Comparison of Slices and Suspensions*— As has been noted by others, the rate of anaerobic glycolysis of brain cortex slices is very variable. For instance, Dickens and Greville (13) give values of $Q_{CO_2}^{N_2}$ between 6.2 and 19.2 for rat brain under comparable conditions. Bumm *et al.* (8) gave values between 3.5 and 10.5. In this work values, over 90 minutes, between 5.7 and 21.5 have been obtained in different experiments, though the majority of the values fell between 10 and 15. The variability was just as marked when the activity was calculated on the basis of initial moist weight as of final dried weight of tissue. No sure correlation between the rate and variations of technique was discovered[4] and the reason for the variability is not yet understood. Duplicate determinations on samples of tissue from the same brains usually agreed within 20 per cent or less. Added pyruvate usually improved the maintenance of rate and reduced the relative difference in activity between samples of slices which varied widely without added pyruvate (see Table IV).

The anaerobic glycolysis of suspensions of whole brain is less variable than that of slices, and duplicate determinations agree exactly. In eleven comparable determinations on different brains, with 100 mg. of tissue in

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[4] The following are some of the variations tested· storing slices till ready in Ringer's solution with or without phosphate, warm or cold, in large or small volume, with or without O_2 bubbling through, or dropping slices directly into the RBG in the manometer vessel; leaving the whole brain dry for 15 minutes, draining slices lightly on a perforated disk or thoroughly on linen, using surface or deeper slices from small or large animals; having the medium freshly treated with 5 per cent CO_2 or exposed to the air, with or without calcium ion in the medium.

3 cc. of RBG, the CO_2 evolution was between 150 and 185 c.mm. in 90 minutes, though no special precautions were taken to equalize the preliminary manipulations. Table IV shows results of experiments in which the anaerobic glycolysis of cortex slices, and of suspensions made from similar slices from the same brains, is compared   The rates for dilute suspensions average nearly the same as for slices, but are less variable; with stronger suspensions the rates per unit weight are lower than with slices.   (Suspen-

TABLE IV

Comparison of Slices and Suspensions

Samples of slices from three brains were placed in manometer vessels containing 2 cc. of Ringer-bicarbonate-glucose (RBG).   The remaining slices were homogenized in RBG and 0.5 cc. of suspension plus 1.5 cc. of RBG or 2.0 cc. of suspension were pipetted into vessels.   Pyruvate, 1 cc. of 0.006 M in RBG, was added from side bulbs. The dry weight of tissue in suspension was calculated from the dry weight of samples of suspension and of RBG, allowance being made for the volume occupied by the tissue in the suspension.

The final dry weight of slices in each manometer was 10 to 16 mg. "Dilute" suspension contained 6 to 9 mg., dry weight, of tissue per manometer; "strong" suspension contained 4 times as much.

C.mm. of CO_2 evolved per mg., dry weight, of rat brain cortex in the first 30 minutes and, in parentheses, in the third 30 minutes.

Slices	Suspension		Slices	Pyruvate, 0.002 M, added		
	Dilute	Strong		Suspension		
				Dilute	Strong	
9.7 (3.9)	8 1 (5 9)	5 1 (3.3)	14.0 (10.6)	9 6 (6 4)	8 0 (5.3)	
11.7 (5 8)			14 7 (11.4)			
4.3 (1.7)	8 5 (3 3)	5.0 (2 9)	10.2 (7.8)	10.5 (6.5)	8.1 (5.3)	
5.2 (2 0)			9.7 (6.9)			
11.4 (5.3)	9.3 (6 3)		11 8 (5.0)		7.8 (4.0)	
10.0 (1.1)			12.3 (7.2)			
8.8* (4.5)	7 9* (5 0)	5.2* (3 1)				

* Average of above and three other experiments.

sions prepared in hypotonic medium show greatly reduced activity, even if salt is added after homogenization.)

Tissue extract inhibited the glycolysis of slices to about the same extent as that of suspensions.   Serum, 2 cc., inhibited the chemically determined lactic acid formation strongly, 60 to 80 per cent.   There was no consistent variation in the rate per unit weight with increasing amounts of slices up to about 20 mg. of final dry weight in 3 cc., but with large amounts, over 40

mg. of dry weight, the rate fell off rapidly with time. Experiments indicated that this effect could be accounted for by accumulation of lactate.

With suspensions, the initial rate of glycolysis is independent of the glucose concentration, at least between 0.001 and 0.017 M. With slices the rate was appreciably lower in 0.0037 M than in 0.0075 M glucose. With suspensions, as with slices (Dickens and Greville (13)), incubation in the absence of both glucose and oxygen causes rapid destruction of the glycolytic activity, 85 per cent in 20 minutes. Addition of pyruvate does not restore the full activity. Traces of oxygen greatly decrease the rate of destruction.

DISCUSSION

In view of the results described in the preceding sections it is apparent that no fixed value can be estimated for the "normal" glycolytic activity of brain and possibly of other tissues. Under conditions of anoxia, the rate at which glycolysis proceeds *in vivo* will be partly determined by the local pyruvate concentration. This, in turn, will depend upon the amount of pyruvate in the circulation or upon the presence or absence of residual traces of oxygen or upon the extent to which pyruvate formed during previous aerobiosis has been destroyed. Further doubt concerning the rates which may occur *in vivo* is raised by the report of Macfarlane and Weil-Malherbe (14) that the rate of glycolysis with slices is very high during the first few minutes of anaerobiosis and by the fact that tissue extracts, properly supplemented, glycolyze very much faster than would be expected from the rates commonly observed with slices or suspensions (Geiger (11); Utter *et al.* (12)). The release of pyruvate-destroying factors and the inhibitory effect of increased tissue concentration may be functions of damage to the tissue. But it is possible that they reflect regulatory mechanisms which may be effective *in vivo*.

Except in the case of muscle, little consideration has been given to the physiological rôle of glycolysis. In muscle it is understood that glycolysis serves as a rapid method for producing energy-rich phosphate bonds when these are used up during activity faster than they can be provided by the oxygen supply and respiration. It is commonly assumed that glycolysis serves a similar function in other tissues. We would like to suggest the possibility that anaerobic glycolysis may be a normal process, not confined to emergency situations in various tissues, and might serve other functions besides the provision of energy. Local fluctuations in oxygen tension probably occur normally in the brain. Chambers and Zweifach (15) have described periodic spontaneous changes in the caliber of the entries to capillaries in various tissues. Davies[5] has observed fluctuations in local oxygen

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[5] Davies, P. W., personal communication.

tension on the brain cortex surface. He found that the oxygen tension varies at different points from the full arterial tension down to at least as low as 5 mm. of Hg. In the preceding paper (3) it was shown that brain tissue respires at its full rate in the presence of very low oxygen tensions, at least as low as 4 mm. of Hg. It seems reasonable to suppose that, if the blood supply to any zone is decreased even briefly, the rapid respiration would quickly reduce the oxygen tension in the neighborhood of respiring cells below the critical level, and the cell group affected would tend to change over from respiration to glycolysis.[6] The rate of glycolysis might be expected to be rapid almost immediately after the oxygen supply is diminished, since, as was shown in the preceding sections, the pyruvate present in the tissue after a period of aerobiosis would produce a nearly maximum glycolysis rate, and traces of oxygen remaining would tend to maintain the high rate. The lactic acid formed within the tissue cells by the glycolysis would be expected to produce marked changes in the functional activity of the cells, owing to the effects of hydrogen ion concentration on the distribution of electrolytes across the cell surface and on the activities of enzymes. Any lactic acid formed, in excess of what could be consumed during early restoration of aerobiosis, would presumably escape from the cells, affect the pH of the surrounding fluids, and tend to produce an increase in the local circulation rate by action on the blood vessels.

Observations in the literature are in accord with the suggestion that glycolysis occurs normally in the brain. Glycolysis in the brain is very rapid once the circulation is stopped by decapitation (18), but lactic acid, which has accumulated in the brain during anoxia, disappears rapidly during recovery, probably by oxidation (19). However, even under normal conditions a small amount of lactic acid is added to the blood stream by the brain (20). Meyerhof and Lohmann (21) showed that fructose will serve as substrate for respiration but not for glycolysis in brain tissue. Since intravenous fructose will not prevent insulin convulsions, they suggested that glycolysis is an indispensable vital function of nervous tissue. Fructose does not maintain the electrical activity of the cerebral cortex in the hepatectomized animal (22).

[6] If the oxygen tension in the tissue is 10 mm. of Hg, there would be present at most 0.3 c.mm. of dissolved oxygen per cc. If the respiration rate of brain is about 5000 c.mm. per gm. per hour (16, 1), the dissolved oxygen could be completely consumed in 0.2 second. Whole unexsanguinated brain contains about 2 per cent of blood (White et al. (17); Elliott, unpublished). If this were all in capillaries it might provide sufficient oxygen to last at most 3 seconds after complete cessation of flow, but the oxygen tension would fall rapidly. (These are average figures for whole brain; the quantities and times would, of course, vary in different regions.)

SUMMARY

Factors affecting the anaerobic glycolysis of brain suspensions have been studied.

1. Maximum stimulation by pyruvate is reached with about 5×10^{-5} M pyruvate.

2. The rate is increased briefly by a preliminary period of aerobiosis, continuously by the presence of a trace of oxygen. These effects are shown to be due to the production, during respiration, of a concentration of pyruvate sufficient to stimulate the glycolysis.

3. The rate is increased by washing the suspension with isotonic medium. This effect is shown to be due to the removal of a system which destroys pyruvate.

4. The rate per unit weight of tissue is decreased by increasing concentration of tissue. This effect is not more than partly due to increase in pyruvate-destroying factors.

5. Added lactate inhibits glycolysis somewhat in the absence but not in the presence of added pyruvate.

6. Serum inhibits considerably. This effect is not due to the destruction of pyruvate.

7. In Ringer-phosphate the lactic acid production is considerably lower than in Ringer-bicarbonate and a large amount of unidentified acid is produced.

8. The activity of cortex slices is much more variable than that of suspensions. The glycolysis rate of dilute suspensions of cortex is nearly the same as the average rate for slices.

9. The possibility that glycolysis is a normal process with functional significance is discussed.

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INFRA-RED ABSORPTION SPECTRA OF STEROIDS

I. ANDROGENS AND RELATED STEROIDS*

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This is the first of a series of papers on the infra-red absorption spectra of steroids, with particular reference to the naturally occurring steroidal hormones.[1] These studies have shown that such spectra can be of considerable aid in the chemical identification of this group of compounds. The present paper contains a description of the methods used in this laboratory for qualitative infra-red spectroscopic analysis of crystalline steroids. The spectra of a number of naturally occurring androgens and related compounds are presented and are discussed in relation to chemical structure.

Methods

Instrument—The instrument used was the Hardy two-beam infra-red spectrophotometer (2), built by Mr. G. Fabian Soderstrom in our shop. Fig. 1 is a schematic diagram of the optical system of the instrument. A Nernst glower (N) is the source of infra-red radiation from which the two beams are derived. One beam passes through a fixed iris diaphragm (D_1) to the mirror M_2, which directs the beam to the top mirror at DM, from which it is focused on the upper portion of the first slit (S_1). The other beam follows a similar course through a variable iris diaphragm (D) to mirror M_1, and then to the bottom mirror at DM, from which it is focused on the lower portion of the first slit (S_1). The sample for analysis is mounted in front of the upper portion of slit S_1 and a blank rock salt plate, or control cell, in front of the lower portion of slit S_1. Beyond slit S_1, the beams pass through an optical system consisting of mirrors M_6, M_3, M_5, M_4, and a sodium chloride prism (P) of 10 cm. base. The prism, through which the beams pass twice, is in a Wadsworth-Littrow mounting. Increments of wave-length in the resultant spectrum are focused on the upper and lower

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portion of a second slit (S_2) through which they pass to a mirror (M_7), which focuses them on two matched thermocouple junctions (J).

The thermocouple junctions are connected in opposition in a circuit containing a Zernike (model Zc) galvanometer. As the prism is rotated, different wave-lengths of infra-red radiation are continuously brought into focus on the thermocouple junctions. When there is no absorption by the sample, there is no difference in the intensity of radiation on the two junctions. However, at wave-lengths at which absorption occurs, the difference of intensity of radiation on the two junctions causes a deflection of the galvanometer. These deflections are amplified on a ground glass scale by a beam of light reflected from the glavanometer mirror.
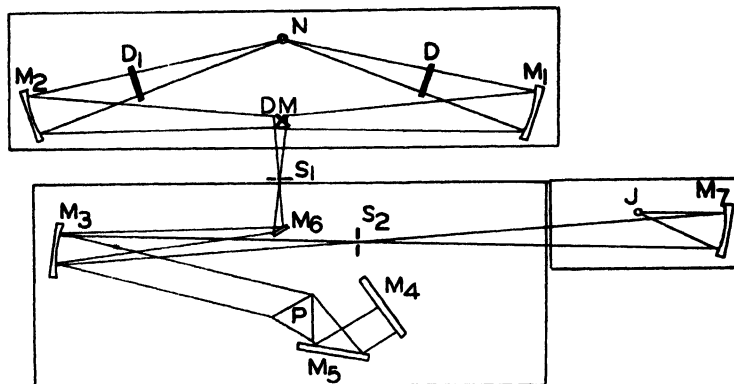
Fig. 1. Schematic diagram of the optical system of the Hardy infra-red spectrophotometer. N is a Nernst glower; D and D_1 iris diaphragms; M_1, M_2, DM, M_3, M_4, M_5, M_6, and M_7 mirrors; S_1 and S_2 slits; P sodium chloride prism; J a pair of matched thermocouple junctions.

The absorption spectrum is recorded by keeping the galvanometer beam at the null point. This is accomplished with a manually operated wheel which controls the opening of the variable iris diaphragm (D) so as to balance the intensities of radiation on the two thermocouple junctions. The wheel also controls a pen which writes on a drum rotated through a gear system by the same motor which rotates the prism. In this manner, as the prism is rotated, the operator obtains a continuous tracing which represents the absorption spectrum of the sample. On each tracing a scale is fixed by means of a vernier attachment on the gear mechanism controlling the prism rotation. This scale, which gives the position of the prism at any point on the tracing, is then converted into a wave-length scale by use of a calibration curve based on absorption spectra obtained with chemical compounds with well defined absorption bands at specific wave-lengths in the infra-red region.

The spectra presented in this paper extend from 2 to 12.4 μ, although the spectrophotometer is capable of analysis out to about 15 μ. From 2 to 4.5 μ, the width of the first slit (S_1) was set at 0.1 mm., from 4.5 to 9 μ at 0.3 mm., and from 9 to 12.4 μ at 0.6 mm. The width of the second slit (S_2) was always set 0.5 mm. greater than the width of the first.

Preparation of Samples—Samples may be investigated either as solid films on rock salt plates or in solution, in suitable cells. In the present paper, only the results obtained with solid films are reported. The films were generally prepared by dissolving the sample in an organic solvent, and then placing the resulting solution dropwise on a salt plate, heated to a temperature suitable for the rapid evaporation of the solvent. When pyridine was used as a solvent, as in most of the preparations reported on in this paper, the salt plate was kept at about 100° during the application of the solution. With heat-stable compounds, crystals were sometimes melted directly on a salt plate and allowed to solidify on cooling.

The most satisfactory films for analysis are either glassy or composed of crystals with little scattering power. When a film tends to deposit in fine, powdery crystals, the scattering effect of the film on the infra-red radiation results in less satisfactory spectra. This difficulty can be partly overcome by grinding the salt plate through which the control beam passes so as to simulate as closely as possible the scattering effect of the film. Usually 4 to 6 mg. of material were used on a salt plate about 2 cm. square and 3 mm. thick; but if material was limited, satisfactory results could be obtained with as little as 1 to 2 mg.

EXPERIMENTAL

The absorption spectra of the following androgens and related steroids are presented in this paper: androsterone, isoandrosterone, dehydroisoandrosterone, 3-chlorodehydroisoandrosterone (Fig. 2); androstanediol-3(α),-17(α), androstanediol-3(β),17(α), Δ^5-androstenediol-3(β),17(α), Δ^5-androstenediol-3(α),17(β) (Fig. 3); androstanedione-3,17, Δ^4-androstenedione-3,17, 3(α)-hydroxyetiocholanone-17, 3(α)-acetoxyetiocholanone-17 (Fig. 4); testosterone, testosterone propionate (Fig. 5).[2]

In the spectra presented, transmission is plotted against wave-length. It should be pointed out that in spectra obtained with solid films, transmission may vary greatly with varying thicknesses and scattering powers of

FIG. 2

different films. Nevertheless, the distribution of absorption bands in the spectrum of any one compound remains constant when different films of that compound are subjected to infra-red analysis.
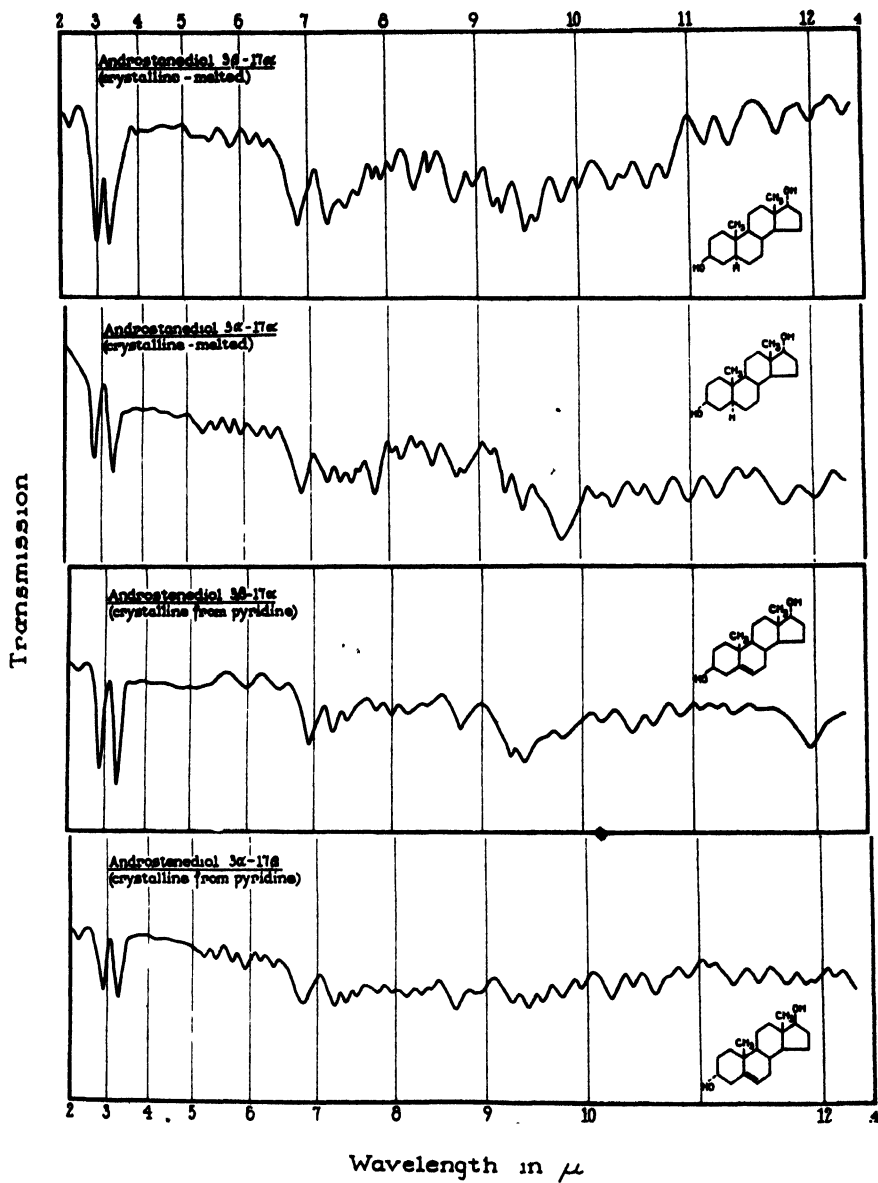
Fig. 3

Analysis of Spectra

Absorption bands in the infra-red region studied here result from interatomic vibrations, whose frequencies are related to the strength of the atomic
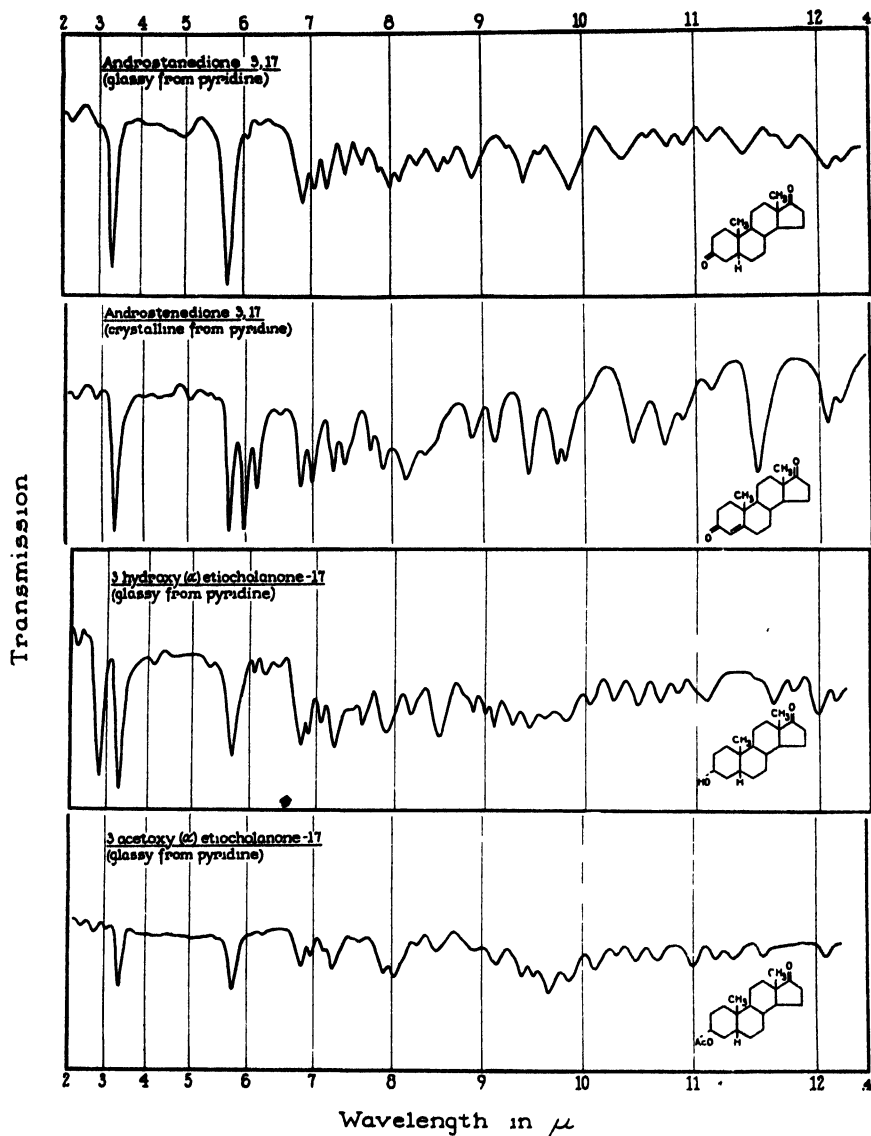
Fig. 4

bonds involved.   It should therefore be possible to assign certain absorption
bands to particular atomic groups or linkages.   This has been accomplished
by comparison of the various spectra obtained in this laboratory as well as
by a study of the infra-red absorption spectra in the literature (*e.g.*, see

Barnes *et al.* (3)).   The absorption bands which can be assigned to specific atomic linkages will be discussed first.

O—H Absorption—A review of the literature (3–6) reveals that absorption bands arising from the linear vibration of hydrogen in alcoholic hydroxyl groups occur between 2.75 and 3 μ.   This rather wide range of absorption has been obtained in studies employing a variety of solvents and concentrations.   However, when alcohols are studied in dilute solutions in carbon tetrachloride or chloroform, the hydroxyl band occurs uniformly between 2.75 and 2.77 μ.   From this it has been inferred that the "free" hydroxyl absorption band occurs at approximately 2.75 μ, and that the broader
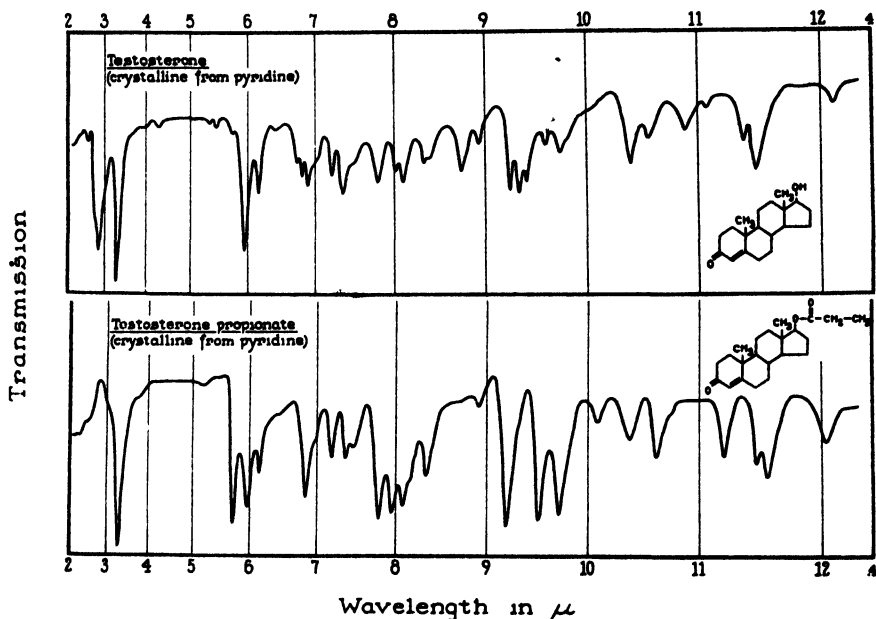
Fig. 5

range of hydroxyl absorption obtained in other than these dilute solutions is due to intermolecular hydrogen bonding.   With the series of solid films studied here, the alcoholic hydroxyl absorption bands were also found to occur over a fairly wide range extending from 2.78 to 2.99 μ.   The concept of intermolecular hydrogen bonding may also provide the explanation for the occurrence of hydroxyl bands at wave-lengths above 2.75 μ in the case of these solid films.   Such bonding would be possible if the hydroxyl groups in these crystalline steroidal preparations were spatially oriented in the proximity of other hydroxyl or carbonyl groups in the crystal lattices.   Evidence for hydrogen bonding of hydroxyl groups in crystal lattices of certain

steroids, such as isoandrosterone and estrone, has been obtained by x-ray analysis (Bernal and Crowfoot (7)). It may be seen in Table I that the extent of the shift of the hydroxyl bands to higher wave-lengths differs for the various members of the series. Since other studies have shown that the extent of the shift to higher wave-lengths is proportional to the strength of hydrogen bonding, the variations noted in Table I are susceptible to the same interpretation. It is possible that in some compounds the orientation of the hydroxyl groups is more favorable for stronger hydrogen bonding than in others.

No absorption bands are present in the hydroxyl region in the spectra of those compounds devoid of hydroxyl groups (androstanedione, androstenedione, testosterone propionate, and $3(\alpha)$-acetoxyetiocholanone-17). It should be pointed out that in the spectrum of 3-chlorodehydroisoandrosterone a small amount of absorption persists in the region near 2.86 μ.

TABLE I

Absorption Maxima in Hydroxyl Region

Compound	Wave length
Androsterone	2 78
Isoandrosterone	2 8.
Dehydroisoandrosterone	2 83
Androstanediol-3(α),17(α)	2 82
Androstanediol-3(β),17(α)	2 99
Δ^5-Androstenediol-3(β),17(α)	2 87
Δ^5-Androstenediol-3(α),17(β)	2.91
3(α)-Hydroxyetiocholanone-17	2 81
Testosterone	2 86

This is regarded as arising from the incomplete conversion of dehydroisoandrosterone to the chloro derivative, the small absorption band in the O—H region arising from the unconverted dehydroisoandrosterone in the sample.

C—H Absorption—In all the spectra presented here, a strong band occurs around 3.35 μ. This band is assigned to the linear vibration of hydrogen in C—H groups of the steroids, since it is in the expected region for absorption of aliphatic C—H groups (3, 8). Aliphatic C—H groups, through an angular vibration of the hydrogen, also produce absorption bands in the region near 7 μ (3). This would account for the bands in the region between 6.75 and 7.00 μ. In some of the spectra, only a single band is found, generally around 6.85 μ, as in the case of androsterone, while in other spectra two bands are resolved, one generally around 6.80 μ, and the other around 6.90 μ, as in the case of isoandrosterone.

CH₃ Absorption—Methyl groups have been found to give rise to absorp-

tion bands in the region between 7.20 and 7.30 μ (3). In all the spectra presented here, a band occurs between 7.20 and 7.25 μ. These bands are interpreted as arising from vibration of the angular methyl groups at C_{10} and C_{13}. In 3(α)-acetoxyetiocholanone-17 and in testosterone propionate, the CH_3 of the acetyl and propionyl groups would contribute to the absorption in this region.

$C=O$ *Absorption*—Carbonyl groups, whether present in ketones, aldehydes, acids, esters, or anhydrides, absorb in the region between 5.45 and 6.05 μ (3). In the compounds under consideration, the following carbonyl groups are encountered: unconjugated ketones at C_3 and C_{17}, conjugated ketones at C_3, and unconjugated ester carbonyl groups. In compounds in which an unconjugated C_{17} ketone is the only carbonyl group present (androsterone, isoandrosterone, dehydroisoandrosterone, 3(α)-hydroxyetiocholanone-17, and chlorodehydroisoandrosterone), the carbonyl absorption band occurs at about 5.75 μ. In androstanedione-3,17 the carbonyl band also occurs at about 5.75 μ; apparently the absorption frequencies of the unconjugated C_3 and C_{17} ketones are so close as to result in only a single large absorption band.

Double bond conjugation of a carbonyl group shifts the absorption to a higher wave-length (3). This is evident in the case of testosterone in which the conjugated C_3 ketone absorption occurs at 5.97 μ. A band at the same position is produced by the same grouping in Δ^4-androstenedione-3,17. In addition, the spectrum of the latter shows a band at 5.75 μ, resulting from the unconjugated C_{17} ketone. Unconjugated ester carbonyls, as found in 3(α)-acetoxyetiocholanone-17 and testosterone propionate, absorb at about 5.75 μ. In the former compound, the ester absorption band is unresolved from the C_{17} ketone band, but in the latter, it is clearly resolved from the conjugated C_3 ketone band at 5.97 μ.

$C=C$ *Absorption*—An unconjugated $C=C$ group has been found to give rise to an absorption band near 6.0 μ (3). Conjugation of the double bond in such a group results in a shift of the band to about 6.20 μ, and generally to an increase in the intensity of absorption (3). Of the steroids under study, dehydroisoandrosterone, 3-chlorodehydroisoandrosterone, Δ^5-androstenediol-3(α),17(β), and Δ^5-androstenediol-3(β),17(α) have an unconjugated double bond between C_5 and C_6. All of these compounds give a weak absorption band between 5.95 and 6.00 μ. In testosterone, testosterone propionate, and Δ^4-androstenedione-3,17, the double bond between C_4 and C_5 is conjugated with the C_3 ketone double bond. To this conjugated $C=C$ linkage can be assigned the band which occurs at about 6.19 μ in the spectrum of each of these steroids.

$C—O$ *Absorption*—Vibration of a $C—O$ linkage when the carbon also has a double bond linkage has been shown to produce an absorption band around 8.0 μ (3). This type of grouping occurs in esters. In 3(α)-acetoxyetio-

cholanone-17, it probably accounts for the band at 8.02 μ and in testosterone propionate, for the band at 7.95 μ.

If the carbon in a C—O linkage has only single bond linkages, the absorption for the C—O vibration occurs between 9.0 and 10.0 μ (3). This type of linkage occurs in all steroids containing hydroxyl groups, and therefore in the spectra of such steroids one might expect to be able to assign bands in that region to the C—O vibrations. However, in any one spectrum there are generally several bands in that region, not only in the case of steroids containing C—O groups, but frequently in the case of steroids having no C—O group (e.g. Δ^4-androstenedione-3,17). This situation necessitated careful cross-comparisons of the spectra of many steroids in the attempt to assign certain wave-lengths of absorption to the C —O linkages of hydroxyl groups. Such cross-comparisons of the spectra in this paper, and also of those spectra to be presented in subsequent papers, have brought out certain relationships between bands occurring in the 9.0 to 10.0 μ region and the presence of different types of hydroxyls in steroids. With respect to certain types of hydroxyls found in the steroids investigated in the present study, the following findings have been made

C_3-(β)-*Hydroxyl trans to a* C_5-*hydrogen*—Steroids with this type of hydroxyl have all given a major band between 9.45 and 9.55 μ (e g., isoandrosterone, 9.47 μ).

C_3-(α)-*Hydroxyl trans to a* C_5-*hydrogen*—Steroids with this type of hydroxyl have all given a band between 9.45 and 9.55 μ. In the case of the 3(α)-hydroxyetiocholanone-17, this band (9.47 μ) is not large, but in certain pregnane and cholic acid derivatives having this type of hydroxyl, the band in this region is a major one.

C_3-(α)-*Hydroxyl cis to a* C_5-*hydrogen*—The only steroids so far examined with this type of hydroxyl are androsterone and androstanediol-3(α),17(α). Both of these compounds give a band of medium intensity near 9.4 μ and a band of strong intensity near 9.9 μ. However, spectra of additional steroids containing this type of hydroxyl are required before bands at either of these wave-lengths can be said with certainty to be related to the C—O group of the type of hydroxyl in question.

C_3-(β)-*Hydroxyl accompanied by a* C_5-C_6 *double bond*—Steroids with this type of hydroxyl have all given a major band between 9.40 and 9.45 μ (e.g., dehydroisandrosterone, 9.41 μ).

C_{17}-*Hydroxyl trans to a* C_{13}-*methyl*— Steroids with this type of hydroxyl have all given a major band between 9.35 and 9.45 μ (e g., testosterone, 9.35 and 9.43 μ (split band)).

If a steroid has two hydroxyl groups included in the types listed above, the absorption resulting from the presence of the two groups is not always resolved into two bands. For example, in the 9.35 to 9.50 μ region, andros-

tanediol-3(β),17(α) gives only one band at 9.47 μ, and Δ^5-androstenediol-3(β),17(α) gives only one band at 9.43 μ.

It is likely that the absorption bands discussed above in relation to different types of hydroxyls in steroid molecules result from the vibration of the C—O linkages of the hydroxyl groups. Even if conclusive proof of this is lacking at the present time, it does not detract from the possible usefulness of these relationships in aiding in the determination of the chemical structure of newly isolated steroids. Finally, it should be reemphasized that major bands sometimes occur near 9.4 and 9.5 μ in the spectra of steroids having no hydroxyl groups. Because of this, it is well to bear in mind that major bands in this region should not by themselves be considered conclusive evidence of the presence of any hydroxyl groups in a steroid.

Other Absorption Bands—It is impossible at present to assign the majority of the absorption bands in the infra-red spectra of steroids to specific interatomic vibrations. Most of these "unassigned" bands occur beyond 10 μ. Some of them between 10 and 11 μ probably arise from C—C vibrations (3). Many of them undoubtedly result from complex vibrations involving the steroid nucleus itself. For this reason, it might be expected that steroids with a similar structural configuration in one part of the molecule would show certain absorption bands in common. With this possibility in view, cross-comparisons were made of the absorption spectra in the region beyond 10 μ. Several apparent relationships between chemical structure and wavelengths of major absorption bands were found. Two of these relationships appear to be well enough established to be reported at the present time.

First, all steroids so far investigated with the configuration A give a
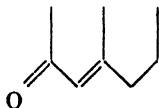
(A)

strong band around 11.5 μ. In Δ^4-androstenedione-3,17, this band is at 11.50 μ, in testosterone at 11.49 μ, and in testosterone propionate at 11.56 μ. As will be shown in a subsequent paper,[3] introduction of a ketone or hydroxyl group at C_{11}, as in certain corticosteroids, tends to lower the wavelength of this major band.

The second relationship involves the configuration B. All steroids

(B)
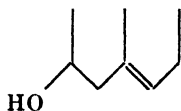
[1] Furchgott, R. F., Rosenkrantz, H., and Shorr, E., unpublished.

so far investigated which have this configuration give a major band near 11.9 μ. In dehydroisoandrosterone, the band occurs at 11.9 μ, and in Δ^5-androstenediol-3(β),17(α) at 11.96 μ. It should be emphasized that this band is one of the bands of major intensity in the spectra of compounds having the structural configuration in question. This point is stressed since nearly all steroids having the structural configurations C give a band of moderate to weak intensity near 12.0 μ.

(C)
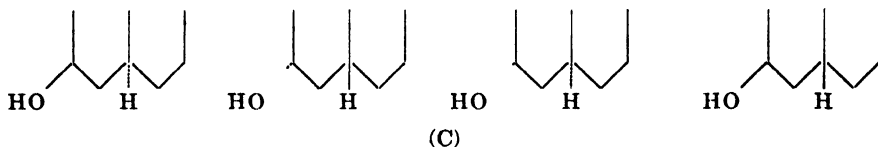
Although many of the bands in the infra-red spectra of steroids cannot as yet be assigned to specific interatomic vibrations or related to definite structural configurations in the molecules, they are nevertheless of great importance in the identification of steroids and in their differentiation by the method of infra-red spectroscopic analysis. Largely because of such bands, this method permits a ready differentiation between steroidal isomers. A comparison of the spectra in this paper shows that this is possible not only for chemical isomers, such as testosterone and dehydroisoandrosterone, but also in the case of steric isomers, such as androsterone, isoandrosterone, and 3(α)-hydroxyetiocholanone-17.

SUMMARY

1. The infra-red absorption spectra from 2 to 12.4 μ of various androgens and related steroids have been presented.

2. Certain absorption bands in these spectra have been discussed in relation to the chemical structure of these compounds.

3. Infra-red spectroscopy constitutes a method of value for the analysis of the chemical structure of steroids.

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A CHICK GROWTH FACTOR IN COW MANURE

I. ITS NON-IDENTITY WITH CHICK GROWTH FACTORS PREVIOUSLY DESCRIBED

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Hammond (1) reported the feeding of dried cow manure to growing chickens. He found that it improved the rate of growth when added to low grade growing diets. The addition of dried cow manure to a good diet did not make it any better. Hammond (2) reported later that dried cow manure supported growth and efficiency of feed utilization in chicks approximately as well as alfalfa leaf meal when added to a practical war time diet containing sardine fish-meal. Whitson *et al.* (3) showed that 8 per cent of dried cow manure and 3 per cent of sardine fish-meal were about equally effective in improving the growth of chicks fed a diet containing 35 per cent soy bean oil meal and no animal protein. These authors concluded that the substance contributed by cow manure was not a protein or any of the chemically characterized vitamins.

This paper shows that the growth substance in manure is not identical with any of the uncharacterized growth factors which have been reported essential for the chick; namely, the *Lactobacillus casei* factors, factor U, factors R and S, vitamins B_{10} and B_{11}, and folic acid.

It has been reported (4) that there are three *Lactobacillus casei* factors. Each factor has been isolated from a different source: one from liver (5), another from yeast (5), and the third from a fermentation residue (4). The evidence thus far reported (6) indicates that the recently synthesized folic acid (Lederle) is identical with the *L. casei* factor from liver. Vitamin B_c, the antianemia and chick growth factor, first reported by Hogan and Parrott (7) and obtained in crystalline form by Pfiffner *et al.* (8), is believed by Stokstad (5) to be identical with the *L. casei* factor from liver.

Two of the three *Lactobacillus casei* factors have been shown to be required by the chick. The factor from liver has been shown to be required (7–11) for the prevention of anemia and the promotion of growth. The evidence presented for the synthetic *Lactobacillus casei* factor (6) shows that the prevention of anemia and the promotion of growth parallel each other when the basal diet is fed alone and supplemented with 50 γ per 100 gm. of diet. The report of Campbell *et al.* (12) shows that vitamin B_c prevents anemia and promotes growth at the same time on a given dose of the crystalline material.

Hutchings et al. (4) stated that the Lactobacillus casei factor from a fermentation residue was "active in the nutrition of the chick." Scott et al. (13) reported that this factor and either α-pyracin lactone or β-pyracin lactone are required for the complete prevention of macrocytic, hypochromic anemia in chicks. The normocytic, hypochromic type of anemia developed when the L. casei factor was supplied without one of the pyracin lactones; the macrocytic, normochromic type of anemia occurred when β-pyracin lactone was added to the diet without the L. casei factor. Daniel et al. (14) have reported on the formation of folic acid (a marked increase in Streptococcus lactis R activity) when the L. casei factor from fermentation residues was incubated with α- or β-pyracin lactone in chick liver brei.

Factor U was first prepared from yeast by Stokstad and Manning (15) in 1938. They presented evidence to show that this factor, when added to their basal diet, stimulated growth of chicks. The growth factor was found to be present in large amounts in alfalfa, middlings, wheat bran, and yeast.

Factors R and S were concentrated from extracts of yeast by Bauernfeind et al. (16) in 1938. The chick's need for factors R and S was confirmed by Hill et al. (17). These workers also showed that factors R and S are not identical with folic acid, Lactobacillus casei factor from liver, or vitamin B_c.

Briggs et al. (18) reported the existence of two water-soluble vitamins needed by the chick. One, essential for proper feather formation, was named vitamin B_{10}; the other, necessary for growth, was named vitamin B_{11}. Both of these vitamins were obtained as concentrates from solubilized liver (Wilson's liver fraction L). Reporting on the distribution of these unidentified vitamins, these authors state, "liver and brewers' yeast are the best sources, adequate at 5 per cent of the diet. Linseed oil meal, soy bean oil meal, alfalfa leaf meal, and grass are comparatively good sources."

Mitchell et al. (19) reported the concentration of a factor (folic acid) from spinach. This factor was active for both Lactobacillus casei and Streptococcus lactis R. Hutchings et al. (4) have reported that the three L. casei factors (from liver, yeast, and fermentation residue) appear to be different from folic acid obtained from spinach. They base this statement on absorption spectra of the four factors. Briggs et al. (20) showed that a concentrate of spinach folic acid obtained from Mitchell's laboratory promoted growth to some extent when added to a basal diet deficient in vitamin B_{11}. This growth was ascribed to vitamin B_{11} activity in the folic acid concentrate. No information has been found in the literature to indicate that the folic acid of spinach is required by the chick. Briggs et al. (18) reported that maximum growth of chicks was obtained on a purified diet containing 17.5 γ of added folic acid per 100 gm. of diet. Hill et al. (17) indicated that the folic acid requirement appeared to be less than 15 γ per 100 gm. of diet. These two figures were not based on spinach folic acid concentrates, but on the activity of the diets for L. casei and S. lactis R.   •

Each experimental group consisted of twenty-five cross-bred chicks. The chicks were reared in electrically heated batteries which were in an air-conditioned room. All experiments were started with day-old chicks and terminated at the end of 6 weeks. The basal diet consisted of yellow corn 38.0 per cent, barley 20.0, alfalfa leaf meal 3.0, soy bean oil meal 35.0, butyl fermentation solubles (containing 250 γ of riboflavin per gm.) 0.6, steamed bone meal 1.5, limestone 1.0, salt (96 per cent NaCl, 4 per cent $MnSO_4 \cdot 4H_2O$) 0.7, and vitamins A and D feeding oil (400 A. O. A. C. units of vitamin D, 2000 U. S. P. units of vitamin A per gm.) 0.2. To each 100 gm. of this diet were added 50 mg. of choline chloride and 1 mg. of nicotinic acid hydrochloride. The various supplements tested replaced an equal weight of corn in the diet.

In order to determine the relationship between the antianemia factors reported in the literature and the chick growth factor in cow manure, a study of the hemoglobin and hematocrit values in 6 week-old chicks was made. The hemoglobin and hematocrit values were determined for twelve chicks that had been reared on the basal diet and for a similar number reared on the diet supplemented with 8 per cent cow manure. The average hemoglobin and hematocrit values for the basal group were 11.0 gm. per cent and 29.1 per cent respectively. These chicks compared favorably with the chicks fed cow manure, whose average hemoglobin and hematocrit values were 11.7 gm. per cent and 30.5 per cent respectively.

Evidently the hemoglobin and hematocrit values for the basal group were normal. The addition of 8 per cent of cow manure to the diet did not make any significant changes in these blood characteristics.

Yeast is a good source of factors U, R, and S, and vitamins B_{10} and B_{11}. Butyl fermentation solubles are a good source of the characterized B vitamins and probably many of the unidentified vitamins. Experiment 1 was designed to determine how the addition of yeast or butyl fermentation solubles to the basal diet would affect the growth of chicks. Also included in this experiment were some groups fed pyracin lactone and some related compounds. Scott et al. (21) reported that pyracin lactone was essential for the promotion of growth and prevention of anemia in chicks. The supplements fed and the average weights of the chickens at 6 weeks are listed in Table I.

The results of this experiment show that yeast[1] and butyl fermentation solubles do not contain any substance which is identical with the chick growth factor in cow manure. Neither pyracin lactone nor the other two

[1] These yeast samples were tested for their content of vitamins B_{10} and B_{11} by Mr. Robert Lillie and Dr. G. M. Briggs, Jr., of the University of Maryland, and were found to be potent sources of both vitamins.

related compounds showed any beneficial effect on growth at a level of 1 mg. per kilo of diet.

Alfalfa leaf meal is a good source of factor U. Solubilized liver is the material from which concentrates of vitamins B_{10} and B_{11} have been prepared. In Experiment 2 the basal diet was supplemented with these materials in order to determine whether they would contribute anything to the growth potential of this diet. The supplements fed and the average weights of the chickens at 6 weeks are listed in Table I.

TABLE 1

Results of Feeding Various Supplements Containing Unidentified Factors

Experiment No	Supplement	Average weight of chickens at 6 wks.
		gm
	None	333 3
	8% cow manure	427 5
	5% butyl fermentation residue	311.5
	5% yeast (Anheuser Busch strain G)	299.7
	5% " (Fleischmann)	312.8
	Pyridoxamine dihydrochloride (1 mg per kilo diet)	289 7
	Pyridoxal hydrochloride (1 mg per kilo diet)	311.3
	Pyracin lactone (1 mg per kilo diet)	295 1
	None	352.0
	5% cow manure	451 2
	5% alfalfa leaf meal	296 8
	3% Wilson's liver fraction L	472 2
	None	214.2
	Folic acid (Lederle synthetic) (1 mg per kilo diet)	215.3
	0.1% choline chloride	239.9
	Folic acid (1 mg per kilo diet) + 0 1% choline chloride	248.6

Alfalfa leaf meal did not contribute any of the deficient growth substance to the basal diet. However, solubilized liver was a very potent source of the substance in which the basal diet was deficient. In this experiment and in all other experiments related to this problem, the feather growth of the chicks on the basal diet was normal.

During the course of these experiments the synthetic folic acid (Lederle) became available. Experiment 3 was planned to determine whether this synthetic compound would have any effect on chick growth when added to the basal diet alone or with an additional 0.1 per cent choline chloride. The supplements fed and the average weights of the chickens at 6 weeks are listed in Table I.

It is evident that synthetic folic acid (Lederle) did not improve the growth-promoting properties of the basal diet.

DISCUSSION

That the growth factor of cow manure is not identical with any of the previously reported growth factors is evident. It improves the growth-promoting properties of the basal diet, whereas the other factors and ingredients containing them do not. Deficiency of the *Lactobacillus casei* factors causes anemia in chicks, which parallels poor growth. The basal diet described here prevents anemia but does not support good growth. Furthermore, synthetic folic acid (Lederle) and pyracin lactone did not stimulate growth when added to the basal diet. These antianemia factors, therefore, cannot be identical with the growth factor of cow manure.

Yeast is a good source of factors U', R, and S, and vitamins B_{10} and B_{11}. Two different yeasts when fed as 5 per cent of the diet failed to show the growth-promoting effect of cow manure. Alfalfa leaf meal, which is a good source of factor U and a fair source of vitamins B_{10} and B_{11}, failed to stimulate growth in these experiments. The basal diet cannot be deficient in vitamin B_{10} because it supports normal feather growth. Thus, the growth factor in cow manure is not identical with factors U, R, or S, or vitamin B_{10} or B_{11}.

Solubilized liver as well as cow manure proved to be a good source of substances which promote growth when added to the basal diet, whereas butyl fermentation solubles did not.

It was reported previously from this laboratory (22) that the growth of chicks fed this same basal diet was stimulated by the addition of 0.1 per cent choline chloride in combination with either 2 mg. of calcium pantothenate or 0.4 mg. of pyridoxine hydrochloride per 100 gm. of diet, although the basal diet was calculated to contain more than adequate quantities of all three of these vitamins. That cow manure does not owe its growth-promoting properties to such a combination of known vitamins is indicated by fractionation experiments to be reported in another paper (23).

SUMMARY

Evidence has been presented to show that the growth factor of cow manure is not identical with the *Lactobacillus casei* factors (from liver, yeast, or fermentation residues), factors U, R, or S, vitamins B_{10} or B_{11}, synthetic folic acid (Lederle), or pyracin lactone.

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A CHICK GROWTH FACTOR IN COW MANURE

II. THE PREPARATION OF CONCENTRATES AND THE PROPERTIES OF THE FACTOR

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In a previous report (1) evidence was presented which showed that a new unidentified growth factor for the chick was present in dried cow manure. This paper is a report on methods for the concentration of the growth substance.

EXPERIMENTAL

An extract was prepared from cow manure in the following manner. Fresh manure containing little or no urine was dried in an oven at approximately 45° for 24 to 36 hours. It was then ground in a hammer mill through a 3/16 inch screen. A Büchner funnel, approximately 25 cm. in diameter, set in a 4 liter vacuum flask, was used for leaching the manure. A No. 2 Whatman filter paper was placed in the funnel, the vacuum turned on, and 450 gm. of ground manure poured in, leveled, and packed lightly around the edge of the funnel. On top of the manure was placed another filter paper, and finally boiling water was poured into the funnel in such a manner that the entire surface of paper and manure became wet almost simultaneously. The boiling water was poured in the funnel at such a rate that there was only a thin film of water over the top filter paper. When this procedure was followed correctly, it was possible to pour about 4.5 liters of boiling water over the manure in 5 to 10 minutes. Even minor variations in the procedure sometimes caused the manure to absorb the first addition of water and hold it, clogging the filter and permitting only a small volume of water to pass through into the flask.

The extract prepared for Experiment 1 was made by extracting the manure twice. After the first extraction, the residue was dried and then extracted again. A sample of 3600 gm. of manure was extracted. The extract contained 444 gm. of air-dried solids and the residue weighed 2883 gm. The apparent loss of 273 gm. might have been due to a change in moisture content and to small losses in handling. An alcohol extract was also prepared with 50 per cent ethyl alcohol at 50°. Again, a sample of 3600 gm. of manure was extracted. The extract contained 468 gm. of air-dried

solids and the residue weighed 3004 gm.   The difference of 128 gm. may be explained by the same reasons previously stated.

The chicks used were day-old Rhode Island Reds.   There were twenty-five chicks to a group.   The chicks were reared in battery brooders in all of the experiments.   The basal diet has been described (1).   The supplements tested replaced an equal weight of corn in the basal diet.   The supplements fed and the average weights of the chickens at 5 weeks of age are listed in Table I.

The results of Experiment 1 show that the growth factor was extracted by boiling water and by 50 per cent ethyl alcohol at 50°.   It is also evident · that the method of extraction was not very efficient; the extracted manure still contained considerable growth potency.

<div align="center">

TABLE I

Growth Tests with Cow Manure Extracts

</div>

Experiment No.	Supplement	Average weight of chickens at 5 wks.
		gm
	None ·	244.0
	8% dried cow manure	272.1
	0.98% aqueous extract of manure	279.6
	6.41% extracted residue of manure	293.0
	0.98% aqueous extract and 6.41% residue	277.8
	1.04% alcohol (50%) extract of manure	273.6
	6.74% " (50%) residue " "	261.4
	1.04% " extract and 6.74% residue	274.6

<div align="center">

Least significant difference 29.5 gm. (19:1 odds)

</div>

In Experiments 2 and 3, extracts of cow manure were made with three organic solvents, 95 per cent ethyl alcohol, chloroform, and ether.   These extracts were made by allowing the dried ground manure to soak for several days in a large excess of the solvent.   The residues were removed by filtering through a Büchner funnel and reextracted in a similar manner.   However, the chloroform and ethyl alcohol extracts were heated to 50° before they were filtered.   The solvents were removed from the extracts by distillation. Chloroform and alcohol were removed at a bath temperature of 50° under reduced pressure.   The chicks were Rhode Island Reds and the rearing procedure was the same as for Experiment 1.   Supplements fed and average weights of the birds at 6 weeks are listed in Table II.

The results of feeding extracts of cow manure made with organic solvents

show that 95 per cent ethyl alcohol was a fair solvent for the growth factor; but, like water, it did not bring about complete extraction. The factor was insoluble in chloroform and ether.

Experiments 5 and 6 were a test of potency of the air-dried aqueous extract and at the same time a determination of some of the properties of the growth substance in the extract. Each experimental group consisted of six 1-day-old Rhode Island Red chicks. Except for the dried manure supplement, which replaced corn in the basal diet, all supplements were added to the basal diet. The supplements fed and the average weights are listed in Table III.

TABLE II

Growth Tests with Fractions Extracted by Organic Solvents

Experiment No	Supplement	Average weight of chickens at 6 wks.
		gm.
	None	294.2
	8% cow manure	388.8
	0.16% ethyl alcohol (95%) extract $\approx$ 8% cow manure	360.8
	7.84% " " (95%) residue $\approx$ 8% " "	406.3
	0.50% chloroform extract $\approx$ 8% cow manure	260.1
	7.50% " residue $\approx$ 8% " "	377.7
	Least significant difference 43.2 gm (19:1 odds)	
	None	305.4
	8% cow manure	435.6
	0.22% ether extract $\approx$ 8% cow manure	302.2
	7.78% " residue $\approx$ 8% " "	400.4
	Least significant difference 42.5 gm. (19:1 odds)	

The heated manure extract was first ground to a fine powder and then heated at 100° in the dry state for 1 hour. The dialysis preparations were made by filling a cellophane tube with the filtrate from cow manure and allowing it to dialyze into distilled water until no color came through. This took several days; therefore, the whole process was carried out in a refrigerator at 10° in order to prevent putrefaction. The manure filtrate solutions could not be kept very long even at refrigeration temperatures, as they became putrid in a few days. Experiment 6 was designed to determine whether autoclaving the filtrate solutions at 120° and 15 pounds pressure for 15 minutes would destroy the growth substance.

Experiment 5 showed that the aqueous extract was potent when fed at a level of 125 mg. per 100 gm. of the diet.   The growth substance in manure was not destroyed when heated dry at 100° for 1 hour.   It did not dialyze through a cellophane membrane.   Evidence was obtained in Experiment 6 that the extract after autoclaving for 15 minutes at 120° and 15 pounds pressure was potent when fed at a level of 0.75 per cent.   Lower levels were not fed.

TABLE III

Potencies and Properties of Extracts

Experiment No	Supplement	Average weight of chickens at 6 wks
		gm.
5	None	282.0
	"	271.8
	"	244.7
	8% cow manure	304.0
	0.125% dried aqueous extract	322.0
	0.25% " " "	336.0
	0.50% " " "	303.0
	0.75% " " "	345.8
	0.125% extract heated at 100°, 1 hr.	325.8
	0.25% " " " 100°, 1 "	214.8
	0.50% " " " 100°, 1 "	323.5
	0.75% " " " 100°, 1 "	278.8
	Manure filtrate dialysate ⇌ 0.75% dried extract	284.0
	" " dialysis residue ⇌ 0.75% dried extract	323.7
6	None	263.2
	"	288.8
	"	279.0
	"	260.8
	Autoclaved extract ⇌ 0.75% dried extract	349.5

During the course of these experiments it had become apparent that the chicks used were not suitable.   The differences between the negative and positive controls were often too small; there was also a great deal of variability among the chicks.   It was decided to test the progeny of hens fed different diets.   There were four hen diets involved; these are tabulated in Table IV.

Diet 311 was an all-plant protein diet containing 30 per cent of soy bean oil meal and in many respects was very similar to the chick basal diet.   Diets 312 and 315 had high levels of fish-meal and Diet 314 was the same as Diet 311, except that 5 per cent of cow manure replaced 5 per cent of yellow corn.

· These diets were fed to cross-bred hens (Rhode Island Red × Plymouth Rock) mated to New Hampshire males. The chicks were tested in Experiment 7. The maternal diets, the diets fed the chicks, and the average weights of the progeny are listed in Table V. In this experiment all chicks were fed the basal diet for the 1st week of life. There were six chicks in each group. On the 8th day they were divided into groups of approximately equal average weights and given the experimental diets.

The results of Experiment 7 indicated that fish-meal and to some extent cow manure in the hen's diet contributed something to the chicks which enabled them to grow as well on the chick basal diet as when the diet was supplemented with 5 per cent cow manure. Diet 311 did not possess this property. In subsequent experiments, only chicks whose dams were fed

TABLE IV

Maternal Diets .

The diets are given in per cent.

Ingredients	Diet 311	Diet 312	Diet 314	Diet 315
Yellow corn	57.0	78.3	52.0	48.5
Alfalfa leaf meal	5.0	5.0	5.0	5.0
Soy bean oil "	30.0		30.0	30.0
Steamed bone "	4.2	3.2	4.2	2.7
Limestone	2.3	2.0	2.3	2.3
Butyl fermentation solubles	0.5	0.5	0.5	0.5
Salt (94% NaCl; 6% MnSO₄)	0.5	0.5	0.5	0.5
Iodized salt	0.2	0.2	0.2	0.2
Vitamins A and D feeding oil	0.3	0.3	0.3	0.3
Sardine fish-meal		10.0		10.0
Dried cow manure			5.0	

Diet 311 were used; they were selected on the 8th day, after having been fed the basal diet for a week.

The fresh manure extracts had a pH range of 6.5 to 7.0. It was noted that when the autoclaved extract was acidified with HCl to pH 3.0, a gelatinous precipitate formed. The major portion of the precipitate came out of solution in the pH range 3.0 to 4.0. Attempts to filter off the precipitate met with failure because of clogging of the filter. However, centrifuging for 30 to 45 minutes proved successful. The fractions were dried to a thick paste in an oven at 45° and then to dryness in a vacuum desiccator. The acid-soluble material was very hygroscopic. A test of these fractions was made in Experiments 8 and 9. The supplements fed and average weights of the chickens at 6 weeks are tabulated in Table VI. There were six chicks in each group.

The results of these experiments showed that the major portion of the activity was precipitated by acid.

The dried acid precipitate fraction was insoluble in distilled water.　How-

TABLE V

Effect of Maternal Diet on Growth Potentialities of Chicks

Maternal diet No	Supplement to chick diet	Average weight of progeny at 6 wks.
		gm.
311	None	363.0
311	"	394.0
311	5% cow manure	494.0
311	5% " "	502.8
312	None	512.5
312	"	524.6
312	5% cow manure	441.3
312	5% " "	477.6
315	None	447.0
315	"	497.0
315	5% cow manure	465.3
315	5% " "	455.8
314	None	416.0
314	"	377.0
314	5% cow manure	475.6
314	5% " "	446.3

TABLE VI

Potency of Fractions of Manure Filtrate

Experiment No	Supplement	Average weight of chickens at 6 wks.
		gm.
8	None	474.8
	5% cow manure	533.6
	0.2% acid solubles (pH 3.0)	489.0
	0.2% " ppt. (pH 3.0)	538.5
9	None	351.2
	"	294.6
	5% cow manure	426.6
	0.05% acid solubles (pH 3.0)	375.8
	0.05% " ppt. (pH 3.0)	430.3

ever, it dissolved slowly when the solution was adjusted to pH 7.0.　This was done by adding a concentrated solution of NaOH dropwise with stirring until neutrality was nearly reached and then continuing to add a more dilute alkali solution.

When an equal volume of saturated $(NH_4)_2SO_4$ was added to a neutral 3 per cent solution of the acid precipitate fraction, a precipitate formed. The half saturated $(NH_4)_2SO_4$ solubles were removed from the precipitate by centrifugation. Most of the original material was precipitated by the $(NH_4)_2SO_4$. A negligible amount of material was removed by washing the precipitate twice with a half saturated solution of $(NH_4)_2SO_4$. The washings and the original solution were combined. The $(NH_4)_2SO_4$ was removed from both fractions by dialysis through a cellophane membrane.

The fractions produced with half saturated $(NH_4)_2SO_4$ were tested in Experiments 10 and 11. The supplements fed and the average weights of the chickens at 6 weeks are listed in Table VII. There were six chicks in each experimental group.

TABLE VII

Potency of Fractions of Acid Precipitate

Experiment No.	Supplement	Average weight of chickens at 6 wks.
		gm.
10	None	373.0
	5% cow manure	457.0
	0.07% acid ppt. fraction	439.1
	Half saturated $(NH_4)_2SO_4$ solubles $\approx$ 0.07% acid ppt.	481.8
	" " " ppt. $\approx$ 0.07% acid ppt.	461.1
11	None	273.5
	0.05% acid ppt. fraction	414.2
	Half saturated $(NH_4)_2SO_4$ solubles $\approx$ 0.035% acid ppt.	402.5
	" " " " $\approx$ 0.07% " "	378.4
	" " " ppt. $\approx$ 0.025% " "	361.4
	" " " " $\approx$ 0.05% " "	345.5

It is apparent from the 6 weeks weights that the growth factor was divided between both fractions. However, since most of the inert material was precipitated by the $(NH_4)_2SO_4$, the growth substance was concentrated in the solubles. This fraction, when fed at levels equivalent to 0.035 and 0.07 per cent of acid precipitate, introduced, respectively, only 3.75 and 7.5 mg. of solids per 100 gm. of diet. Therefore the $(NH_4)_2SO_4$ solubles constituted only 0.00375 or 0.0075 per cent of the diet. Experiment 11 was a repetition of Experiment 10. The same half saturated $(NH_4)_2SO_4$ preparations were tested at different levels of the diet.

DISCUSSION

Whitson *et al.* (2) showed that cow manure contained a substance which stimulated the growth of chicks and was distinct from the characterized

vitamins.   This factor was shown by Rubin and Bird (1) to be different from the previously described growth factors required by chicks.

In this report a method for extracting the new growth substance from cow manure is presented.   The growth factor was found to be soluble in water, 50 per cent ethyl alcohol, and 95 per cent ethyl alcohol; it was insoluble in chloroform and ether.   It was stable in the dry state at 100° for 1 hour, and was not destroyed by autoclaving in solution for 15 minutes at 120° and 15 pounds pressure.   It did not dialyze through a cellophane membrane.   It was precipitated from solution at pH 3.0.   A method of concentrating the growth factor has been developed.   Optimum growth was obtained when only 3.75 to 7.5 mg. of the concentrate were added to 100 gm. of diet.

It has been pointed out (1) that the growth of chicks fed the basal diet was stimulated by feeding high levels of calcium pantothenate or pyridoxine hydrochloride in conjunction with 0.10 per cent of additional choline chloride.   The growth-promoting activity of the cow manure concentrate cannot be ascribed to these vitamins.   The total weight of the pure vitamins required exceeded the amount of the crude concentrate which was active at 3.75 to 7.5 mg. per 100 gm. of diet.

Hens that were supplied with dried cow manure or fish-meal transmitted enough of the growth substance to their chicks so that the chicks had more than an ample supply in their bodies to last at least 6 weeks, and hence were able to grow well when fed the basal diet.

SUMMARY

1. A method of extracting and concentrating the growth factor of cow manure has been developed.   The factor stimulates the growth of chicks fed a practical diet free of animal protein.   The most potent concentrate obtained supported optimum growth when fed at levels of 3.75 to 7.5 mg. per 100 gm. of diet.

2. The factor is stable to heat in the dry state at 100° for 1 hour and to autoclaving in solution for 15 minutes.   It will not dialyze through cellophane.   The factor is moderately soluble in water, 50 per cent ethyl alcohol, and 95 per cent ethyl alcohol; it is insoluble in chloroform and ether.

3. The factor can be transmitted from the hen through the egg to the chick.

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CARBOHYDRATE METABOLISM IN THE ASCORBIC ACID-DEFICIENT GUINEA PIG UNDER NORMAL AND ANOXIC CONDITIONS

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Although there has been much effort expended in the study of the possible rôle of ascorbic acid in carbohydrate metabolism, this relationship remains obscure.

Sigal and King (1) showed that the glucose tolerance was markedly lowered in a group of guinea pigs deficient in ascorbic acid for 10 days, that the tolerance fell further through the 20 day period during which the deficiency was maintained, and that treatment with ascorbic acid returned the tolerance to normal in 15 days. Altenberger (2) noted that the liver glycogen of scorbutic guinea pigs was lower than that of normal animals. Giroud and Ratsimamanga (3) also found liver and muscle glycogen in ascorbic acid-deficient guinea pigs lowered in direct ratio to the duration of the deficiency and the muscle phosphocreatine decreased. Nair (4) reported decreased glucose tolerance and liver glycogen in scorbutic guinea pigs. Hamne (5) obtained evidence with pair-fed guinea pigs that in chronic scurvy the glycogen content of the liver and muscle was lower than in the normal animal. The liver glycogen was decreased in the early stages of scurvy and the muscle glycogen at a later stage, while the glycogen of the heart was not affected.

Involvement of the adrenal and thyroid glands in the changes incident to ascorbic acid deficiency has been suggested. La Mer and Campbell (6) first reported increased size of the adrenals in scorbutic guinea pigs, and this has been confirmed by several other investigations (7–9). The most recent report is that of Baldwin, Longenecker, and King (10), who found in a series of twenty-five pairs of matched guinea pigs that the average weight of the adrenal glands of the normal animals was 182 mg. and that of ascorbic acid-deficient animals 257 mg. The earlier literature contains many reports of degenerative and congestive changes, including lipid deposition, in the adrenals of scorbutic guinea pigs, but in nearly all of these studies the effect of inanition was not ruled out. MacLean, Sheppard, and McHenry (11) compared the tissues of scorbutic guinea pigs with those of normal animals which had been restricted to the reduced food intake of

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the deficient group and found by microscopic examination no differences in the adrenal glands. Baldwin, Longenecker, and King (10), likewise using paired animals, found increased size of the adrenals of the deficient animals but no changes in their lipid content.

No studies of the effect of ascorbic acid deficiency upon the behavior of animals under anoxia could be found. When Sacerdote (12) subjected normal guinea pigs and rabbits to mixtures of nitrogen and oxygen in which the concentration of the latter was gradually reduced to 3 per cent in 10 hours, there occurred a notable increase in the ascorbic acid level of the blood and a decrease in the ascorbic acid content of the adrenals. No observations were made on the carbohydrate metabolism.

Wertheimer (13) found no change in the blood sugar of guinea pigs, presumably normal, kept for 3 to 11 days at 340 mm. pressure, little change in the liver glycogen, and none in the muscle glycogen.

The response to anoxia of pair-fed normal and scorbutic guinea pigs might be expected to offer some index of the condition of the adrenal glands, since it is now believed (14) that adaptation to anoxic conditions is dependent on the mediation of that gland.

Several reports of abnormal oxygen consumption in the later stages of scurvy have appeared. Mosonyi and Kézdi (15) attributed this to hyperactivity of the thyroid, as did Hamne (5). Spence and Scowan (16), however, found no hyperplasia of the thyroid in acute scurvy and not in all cases of chronic scurvy. Törnblom (17) reported diminished oxygen consumption toward the end of the deficiency state. These contradictory observations made it seem worth while to study the oxygen consumption of pair-fed normal and scorbutic guinea pigs.

This experiment was undertaken to determine the extent of carbohydrate absorption, blood sugar levels, glycogen content, and oxygen consumption of normal and scorbutic guinea pigs under normal atmospheric pressure, and their utilization of carbohydrate under moderate anoxia. Some observations were also made on the weights of adrenal glands of normal and severely deficient animals and on the lipid content of livers and carcasses.

EXPERIMENTAL

Young guinea pigs (350 to 400 gm.) of both sexes were used. They were obtained from the laboratory colony and were paired carefully as to weight, sex, and litter origin. One of each pair was given no ascorbic acid but was allowed to consume the basal diet *ad libitum*. Each day the amount consumed was ascertained and the normal member of the pair allowed only that amount on the succeeding day.

The basal diet was a commercial rabbit food[1] which has been found satisfactory for the stock colony when supplemented with ascorbic acid. In addition to the feed, the animals were each given 5 gm. of wheat germ and 0.5 gm. of gray fish oil (20,000 units of vitamin A per gm.) weekly. The normal animals were given by pipette 10 mg. of ascorbic acid three times a week. All the animals were kept at a temperature of 25–30°.

The animals kept on this diet without ascorbic acid supplements developed the first loss of appetite usually oh the 14th day and definite scurvy in 19 to 24 days, as was evidenced by extensive hemorrhages of the fascia of the musculature, particularly of the legs. The deficient animals often lived 35 to 40 days or longer after the ascorbic acid supplement was removed from the diet. The progress of the deficiency varied so that guinea pigs, maintained different lengths of time on the basal diet, presented similar stages of scurvy, as manifested by loss of appetite and of weight, inactivity, soreness of joints, and unkemptness of fur. The attempt was made to institute the final determinations on all the animals at the same stage of the deficiency so far as this was possible. Because of the abrupt loss of weight of the scorbutic animals within a day or two of the onset of the symptoms, the final weight of the normal members of the pairs was usually 50 to 60 gm. the greater.

When the deficient member of the pair was judged to be in an acute but not critical stage of scurvy, an event which usually occurred between the 18th and 27th days on the diet, the carbohydrate utilization technique of Cori and Cori (18) was applied at once to both members of the pair. The animals were allowed to fast for 24 hours, were then given orally 5 ml. of a solution containing 2.5 gm. of glucose, and were sacrificed 6 hours later by injection of 1 ml. of 6 per cent sodium amytal intraperitoneally.

Blood was taken by heart puncture. The liver was weighed, transferred at once to a weighed, glass-stoppered Erlenmeyer flask containing 30 ml. of hot 50 per cent KOH, hydrolyzed by heating in a boiling water bath, cooled, and weighed. The gastrointestinal tract was removed, the carcass weighed, plunged at once into a beaker containing 350 to 400 ml. of boiling KOH, and boiled until hydrolysis was complete.

Glucose absorption was determined by the method of Cori and Cori (18), but only the small intestine was used, since even after fasting 24

[1] Globe A-1 Wonder rabbit pellets, distributed by the Globe Grain and Milling Company, Oakland, California, and made of alfalfa meal, ground barley, ground oats, soy bean meal, wheat shorts, wheat bran, molasses, linseed meal, limestone, strained bone meal, salt, and dried whey. It contained, in per cent, crude protein 15.0, fat 2.5, fiber 19.0, ash 10.0, added salts 1.0. and, in mg. per cent, thiamine 0.28, riboflavin 0.65. carotene 0.58.

hours the large intestine of the guinea pig still contained considerable amounts of organic matter. Trials on several animals showed that no glucose was present in the large intestine.

The blood proteins and the protein in the digestive contents were precipitated by the method of Somogyi (19). The blood sugar and the reducing value of the digestive tract were determined by the use of ceric sulfate, according to the method of Giragossintz, Davidson, and Kirk (20). Glycogen in liver and carcass was determined by the method of Good, Kramer, and Somogyi (21). Titration of the resultant reducing solution was carried out with ceric sulfate, and the reducing value of all titrations expressed as glucose.

In a preliminary experiment five pairs of animals were used to determine any differences as to urinary excretion of sugar. The normal animals excreted 115 mg. of reducing substance during 24 hours without food and the ascorbic acid-deficient animals 106. When 2.5 gm. of glucose were given and the urine collected for 6 hours, the excretion of the groups was 45 and 35, or 180 and 140 mg. of glucose in 24 hours. Since the fasting excretion did not differ markedly from that following sugar feeding, true glycosuria was assumed to be absent and it was concluded that no significant effect could be attributed to the deficiency. Similar comparisons were not made, however, in the anoxia experiment.

For the anoxia experiment the animals were also pair-fed. When the appetite of the scorbutic member of a pair declined, both animals were placed in the low pressure chambers and kept there without food for 24 hours. No acclimatization was attempted, but the reduction in pressure was accomplished gradually. The previous fast and glucose feeding used in the glucose utilization experiments were omitted, as was also the analysis of gastrointestinal contents. The animals were sacrificed upon removal from the chambers and blood sugar, liver glycogen, and body glycogen determined.

The low pressure apparatus[2] consisted of a series of 2 liter glass jars connected with the evacuating system by copper tubes sealed in the lids. The air inlet and outlet of the evacuating line were provided with a needle valve which allowed regulation of the adequately rapid air flow through the chambers. A calibrated mercury manometer inserted in the evacuating line was used to indicate the pressure maintained, 349 mm. of Hg, corresponding to 20,000 feet altitude (22).

Results

Glycogen and Blood Sugar, after Glucose Ingestion—In Table I are given the results obtained with three groups of animals. The differences be-

tween the normal and ascorbic acid-deficient animals were of the same order in all the groups. It is evident that the glucose tolerance of the deficient guinea pigs was lowered since their blood sugar was significantly greater than that of the normals in all cases. The mean glycogen content of the normal animals was in all three groups greater than that of the deficient group. This applied both to the absolute quantity in the liver and carcass and to the proportion per 100 gm. of liver and of carcass. The variability of these values was large, however, and the significance of the difference not evident, except in Group III, the scorbutic members of which

TABLE I

Carbohydrate Levels of Normal and Ascorbic Acid-Deficient Guinea Pigs 6 Hours after Glucose Ingestion .

Experimental group No.		No of animals	Glucose fed after 24 hrs fast	Glucose of gastro-intestinal tract	Liver glycogen	Body glycogen	Blood sugar	Body weight
			gm	*mg.*	*per cent*	*mg per cent*	*mg per cent*	*gm.*
I	Normal	5	2.57	15 ± 2	4.44 ± 0.90	383 ± 26	74 ± 4	496 (426–540)
	17–20 days deficient	5	2.57	51 ± 9	3.06 ± 0.52	300 ± 32	101 ± 7	436 (374–490)
II	Normal	9	2.59	17 ± 2	2.81 ± 0.27	379 ± 17	74 ± 4	498 (454–530)
	19–36 days deficient	11	2.59	102 ± 31	2.12 ± 0.23	333 ± 26	112 ± 10	433 (352–470)
III	Normal	9	2.66	58 ± 20	4.07 ± 0.20	362 ± 14	92 ± 6	462 (380–510)
	21–27 days deficient	9	2.66	166 ± 41	2.66 ± 0.33	311 ± 19	162 ± 20	404 (326–450)

were in a more advanced and uniform stage of deficiency than were those of Groups I and II.

The absorption of sugar was reduced in the deficient guinea pigs, as was indicated by the larger reducing value of their gastrointestinal tracts. That absorption was complete in 6 hours in the case of the normal animals was ascertained by allowing twelve normal guinea pigs to fast 24 hours, after which they were sacrificed and the reducing value of the gastrointestinal tract determined. The average content was 17 mg. of glucose, about the same quantity found in the normal animals (Table I) 6 hours after glucose had been given. The difference in absorption between normal and deficient animals was consistent and significant, but not striking when the difference in weight of the animals is considered. Lowered in-

testinal absorption has been observed in various other conditions, in adrenalectomized, fasting, and in thiamine-, riboflavin-, or pantothenic acid-deficient animals.

These differences (Table I) in blood glucose and glycogen levels between normal and scorbutic guinea pigs are in agreement with those described in earlier reports (1–5).

Carbohydrate Metabolism under Anoxia—The differences in carbohydrate utilization between the normal guinea pigs fasting 24 hours at sea level and at 20,000 feet altitude were not as striking as were those found by Evans (23), Lewis *et al.* (14), and others for rats. The blood sugar level was significantly raised, but the liver glycogen was not significantly increased and the carcass glycogen appeared to be significantly decreased (Table II).

TABLE II

Effect of Anoxic Anoxia on Carbohydrate Levels of Fasting Normal and Ascorbic Acid-Deficient Guinea Pigs

Experimental group	No of animals	Liver glycogen	Body glycogen	Blood sugar	Body weight
		mg per cent	*mg. per cent*	*mg per cent*	*gm*
Normal, sea level	6	414 ± 17	307 ± 11	81 ± 4	469 (370–540)
" 20,000 ft. (349 mm. Hg)	12	433 ± 8	257 ± 17	109 ± 6	441 (400–570)
Deficient, sea level	6	55 ± 4	230 ± 6	84 ± 4	428 (370–500)
" 20,000 ft. (349 mm. Hg)	12	610 ± 14	308 ± 23	118 ± 9	410 (350–570)

The deficient animals also had increased blood sugar levels under the reduced atmospheric pressure and significantly increased liver glycogen and carcass glycogen. The levels of glycogen deposits attained were, however, in no case comparable with those seen by Lewis *et al.* (14) in normal rats and rabbits and by Wickson and Morgan (24) in rats under similar conditions.

Size of Adrenals—Thirteen pairs of animals which had been pair-fed for 19 to 27 days were used to determine the relative weights of the adrenal glands. The body weights of the normal guinea pigs ranged from 400 to 510 gm. with a mean of 440 ± 6 gm. and those of the deficient animals from 372 to 442 with a mean of 418 ± 2 gm. In all but two of the pairs the weight of the adrenal glands was greater in the deficient than in the normal member, the mean of the former being 294 ± 8 mg. and of the latter 249 ± 7 mg. The weight of the adrenals of the deficient animals was 0.71

mg. per gm. of body weight and of the normal 0.57 mg. This is similar to the observations recorded by others (10).

Fat Content of Livers and Carcasses—Since all the scorbutic guinea pigs lost weight rapidly as soon as the deficiency was established, it was of interest to determine whether this was due chiefly to fat or water loss. As is shown in Table III, total carcass lipids, determined on aliquots of the hydrolysates (25), were nearly the same in all the groups, deficient and normal, whether exposed to anoxia or not. The liver lipids similarly determined were increased in the anoxic groups, but again there was no significant difference between the normal and scorbutic animals. Sundstroem and Michaels (26) found that rats exposed to low pressures developed fatty yellow livers, a condition duplicated in these guinea pigs.

TABLE III

Liver and Carcass Lipid Content of Normal and Ascorbic Acid-Deficient Guinea Pigs

Condition	No. of animals	Body weight	Carcass*		Liver	
			Weight	Lipids	Weight	Lipids
		gm.	gm	per cent	gm.	per cent
Normal, no anoxia	5	478	380	11.2 ± 0.4	16.5	8.0 ± 0.8
Deficient, no anoxia	4	440	357	12.2 ± 1.1	17.5	7.2 ± 0.5
Normal, exposed to anoxia†	12	434	361‡	12.5 ± 0.7	18.1	13.5 ± 0.6
Deficient, exposed to anoxia†.	12	416	350‡	14.3 ± 0.9	20.0	11.5 ± 0.5

* Body minus gastrointestinal tract and liver.

† 24 hours at 349 mm. of Hg.

‡ Eight animals only used for carcass analysis.

Because it has been shown (27) that scorbutic guinea pigs in these early stages of the deficiency remain in nitrogen equilibrium, it may be conjectured that protein catabolism is not the cause of the abrupt weight loss, and since there was no demonstrable change in fat content of the body the loss may probably be ascribed to dehydration.

Baldwin, Longenecker, and King (10) have also found that in normal and ascorbic acid-deficient pair-fed guinea pigs the gross amounts of lipids in livers and carcasses were alike.

Oxygen Consumption in Ascorbic Acid Deficiency—The oxygen consumption and carbon dioxide production measurements were made by Dr. Max Kleiber,[3] with his apparatus designed for metabolism work with small animals (28). Two series of tests were run, the first on animals fed *ad libitum*, the second on pair-fed groups. The animals were fasted 15 hours

[3] College of Agriculture, University of California, Davis.

previous to the tests, during which time they were kept in an air-conditioned room at a temperature of 30° with free access to water. The test period was 5 hours in length. Two groups of guinea pigs of seven pairs each were used. The body weights were nearly the same, since the deficiency had been in effect only 15 to 30 days. The oxygen consumption of the normal animals corresponded to the production of 36 calories per day or 106 and 89 calories per kilo. The energy output of the deficient animals was 40 and 38 calories per day per animal or 100 and 92 per kilo. The respiratory quotients were the same, 0.74 to 0.78 for all groups. The calories per day per kilo[1] were 80 ± 3 and 71 ± 2 for the normal groups, and 79 ± 4 and 73 ± 1 for the deficient groups. Thus, there was no difference in energy output between normal and deficient animals. The conclusion may be drawn that the abnormality in carbohydrate metabolism observed in this study was not due to deranged thyroid function. This conclusion was borne out by the histological findings. Microscopic examination of sections of the thyroid of the deficient animals gave no indication of hyperplasia of the epithelium or other abnormality.

DISCUSSION

Under the conditions of this experiment, under normal oxygen tension, ascorbic acid-deficient guinea pigs appeared to be somewhat less able to form glycogen than the pair-fed control animals. This might indicate excess epinephrine secretion in the deficient group, as has been suggested by Banerjee (29), or participation by the ascorbic acid in the process of glycogenesis or in the prevention of glycogenolysis.

Under reduced oxygen tension for 24 hours the fasting normal animals were unable to increase their liver glycogen and actually lost muscle glycogen. But the deficient group under these circumstances definitely increased both liver and body glycogen. This may indicate exhaustion of epinephrine or dampening of its effect (30, 31) in the deficient animals under anoxia and stimulation of glyconeogenesis through mediation of the adrenocortical hormone (14). The normal animals apparently resisted these changes, possibly because ascorbic acid exerts a protective effect upon the secretory activity of both the adrenal medulla and cortex (29). Glycogenesis under sea level conditions was favored by the presence of ascorbic acid but glyconeogenesis under anoxia was prevented thereby. The effect may be direct or through changes in the activity of the adrenal secretions.

A comparison of the fasting metabolism of normal and riboflavin-deficient rats by the same technique indicated lowered blood sugar and liver and body glycogen at ordinary pressure in the deficient animals and little increase in carbohydrate levels under anoxic anoxia (24). The normal rats responded under anoxia with increased blood sugar and liver glyco-

gen. Apparently the mechanisms affecting carbohydrate utilization through riboflavin are different from those affected by ascorbic acid.

SUMMARY

Ascorbic acid-deficient guinea pigs were found to have significantly higher blood sugar and probably significantly lower liver and carcass glycogen than the pair-fed normal animals 6 hours after they were fed glucose following a 24 hour fast. The intestinal absorption of the sugar was less complete in the deficient animals.

When similarly paired but fasting animals were exposed to anoxic anoxia, 349 mm. of Hg for 24 hours, the deficient group was able to maintain or increase blood sugar and glycogen stores more effectively than did the normal. Fasting deficient guinea pigs at ordinary atmospheric pressure had lower carbohydrate stores than did those under anoxia, but this was not true of the normal animals.

The ascorbic acid-deficient guinea pigs were found to have a significantly increased size of adrenals, as compared with their paired controls.

The lipid content of livers and carcasses of the deficient animals was found to be nearly the same as in the normal, whether the animals had been subjected to anoxia or not. The sudden loss of weight in the former is therefore probably due to dehydration. The liver lipid values were increased in both groups after exposure to anoxia.

Oxygen consumption was not altered by the ascorbic acid deficiency, and no changes were seen in the thyroids.

The reduced glycogenesis and increased blood sugar exhibited by ascorbic acid-deficient guinea pigs following glucose feeding accord with the theory that the adrenomedullary mechanism is hyperactive in this deficiency. The increased size of the adrenals, rapid dehydration, and glyconeogenesis of the deficient animals under anoxia point to similar compensatory hyperactivity of the adrenocortical mechanism.

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THE EFFECT OF VOLUNTARY OVERBREATHING ON THE ELECTROLYTE EQUILIBRIUM OF ARTERIAL BLOOD IN MAN

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Although there are numerous reports concerning the effect of hyperventilation on several of the constituents of blood, there has not yet been a comprehensive study of the changes in its electrolyte structure. In the earliest studies the alveolar CO_2 tension, the carbon dioxide absorption curve of the blood, and the calcium and phosphorus contents of serum were determined (1). The results seemed to indicate that with rapid fall of the CO_2 tension during hyperventilation the pH may rise to values between 7.7 and 7.8 without changes of the CO_2 absorption curve of blood, while the calcium content of serum remains unchanged and the inorganic phosphorus decreases. These high, indirectly determined pH values have been criticized by Peters and his coworkers (2) on the basis of analyses on the blood of the antecubital vein of one subject, which showed a pH value of only 7.59 during hyperventilation. These observers also determined the chloride and the protein contents of serum. They found a decrease in both the chloride and the total determined value of anions, an unexpected result in view of the expected reciprocal changes in bicarbonate and chloride, and suggested that lactic acid accumulation caused by muscular spasm was responsible for their findings. Another contribution to this problem was made by Gollwitzer-Meier and Meyer (3), who determined in the arterial blood of three subjects the CO_2 content and combining power, and in their serum the conductivity, sodium, calcium, and chloride. With decreases in the CO_2 tension they found diminutions in the CO_2 content up to 7.5 milliequivalents per liter, and they calculated increases in pH values as high as 7.63, while the CO_2 combining power remained unchanged. The conductivity decreased on the average by 1.1 per cent, while the calcium did not change. The serum sodium and chloride varied erratically in their experiments. In a later extensive study on capillary blood Shock and Hastings (4) determined the serum pH and CO_2 content. They found that the CO_2 tension decreased rapidly during the first 3 minutes of hyperventilation and somewhat more slowly thereafter. It reached an average minimum value of 14 mm. of mercury after 20 minutes, with a corresponding peak value of

7.7 for the pH.   After the hyperventilation was discontinued, the values returned to normal at a very rapid rate during the first 2 minutes and at a decreasing rate subsequently, complete recovery requiring 15 to 25 minutes. The bicarbonate content decreased during hyperventilation at a rate of 0.2 to 0.7 milliequivalent per liter per minute, the maximum decrease varying from 4 to 7 milliequivalents.   In the early stages of overbreathing the displacement of the acid-base balance proceeded along the path of the CO_2 absorption curve of blood, while after longer periods there was evidence of accumulation of fixed acid.

The most recent study, concerned with the changes in arterial and internal jugular blood during brief periods of hyperventilation, was carried out on three subjects by Nims and his coworkers (5).   They found that the arterial pH increased rapidly during the first 2 minutes at a rate of 0.1 unit per minute to values as high as 7.67 at the end of 5 minutes, while the changes in the venous blood were of smaller magnitude and proceeded at a slower rate, so that the arterial-venous difference increased during hyperventilation.   The CO_2 content decreased markedly in the arterial blood, but to a small extent only in the jugular blood.   The arterial lactic acid increased by 0.2 to 0.5 milliequivalent per liter during the 5 to 6 minute periods of hyperventilation, while the corresponding change in venous blood was 0.3 to 0.6 milliequivalent.

The effect of pH on the distribution of the electrolytes in blood has been studied by several observers *in vitro* in connection with tests of the validity of the Donnan theory concerning the water and electrolyte equilibria on two sides of a semipermeable membrane.   The pioneering study which embodies the most comprehensive theoretical treatment of the problem was carried out by Van Slyke, Wu, and McLean (6).   They found that the distribution of electrolytes in blood cells and serum followed in a general way Donnan's theory.   Later studies, while providing several corrections in the numerical values of their estimates, have tended to support the main thesis (7, 8). It appeared to us of particular interest to study voluntary hyperventilation with a view to testing the effect of pH changes on the distribution of electrolytes *in vivo*, because of the great variations expected, the rapidity of the effects, and their reversibility.   Under such conditions the changes in electrolyte distribution should be primarily dependent upon variations in the ionic equivalency of hemoglobin, in contrast to the results of a previous study on the electrolyte distribution during pyloric obstruction, where changes in the organic phosphates predominated (7).

That the chemical effect of hyperventilation is not limited to changes in the electrolyte distribution is indicated by the finding that the inorganic P of plasma decreases, and ketonuria occurs.   The physiologic changes are not confined to the occurrence of tetany, but also include changes in con-

sciousness, which are associated with variations from the normal in the electroencephalogram (9). Since such effects may well be associated with changes in the electrolytes, it was decided to include in this study the determination of the inorganic P and potassium content of the serum. The interrelation between the pattern of the electroencephalogram and the changes in the electrolyte structure of the blood will be discussed elsewhere.

EXPERIMENTAL

Seven normal men served as experimental subjects. The subject came to the laboratory, without breakfast, at 8 a.m., undressed, and stretched out on a bed. Half an hour later a needle fitted with an obturator was placed in the femoral artery under local anesthesia. Because of the painful stimulus of the procedure and the anxiety of the subject involuntary overbreathing occurred on several occasions. After an interval of several minutes, to permit restoration of the basal condition of the subject, sampling was begun. The blood was collected by means of a tight fitting syringe containing paraffin oil. The physiologic effects of the overbreathing, as described by previous observers, included flushing of the face, numbness and tingling of the face and extremities, decreased level of consciousness, and occasionally tetany with carpopedal spasms. Electroencephalograms were obtained on the majority of the subjects.

Four to six blood samples were withdrawn from each of the subjects, one or two before the period of hyperventilation started, two during the period of overbreathing approximately 3 and 6 minutes after the start, and one or two more 3 and 6 minutes after cessation of the overbreathing. For control purposes five samples of blood were drawn from one of the subjects at rest under conditions otherwise simulating an actual experiment. In all, sixteen samples of blood were obtained before, fourteen during, and eleven after the periods of hyperventilation.

The blood samples were distributed as follows: one portion, for the determination of oxygen content and of the CO_2 in the whole blood, was delivered over mercury into a vessel containing 0.1 cc. of a 30 per cent solution of potassium oxalate; another portion, for the various analyses to be performed on serum, was transferred under paraffin oil to a centrifuge tube; and a third portion, for the whole blood analyses which did not require protection from exposure to air, was put into a vial containing dried heparin. The following constituents were determined in the whole blood: dextrose, oxygen and CO_2 content, chloride, hemoglobin, and the volume of packed cells. The measurements on the serum included the determination of pH, CO_2 content, chloride, sodium, and inorganic P on all of the subjects, and of potassium and calcium on four of them. From the data on pH and CO_2 content the CO_2 tension was calculated by means of the Henderson-Hassel-

balch formula. The cellular concentrations of CO_2 and chloride were calculated, by means of the cell volume, from values determined on whole blood and serum. The hemoglobin concentration in the cells was computed by dividing the whole blood value by the relative cell volume. The methods used have been described previously (7, 10).

The concentration of H_2CO_3 in the cells was calculated by the use of the solubility coefficient of carbonic acid given by Van Slyke *et al.* (11) under the assumption that the CO_2 tension was the same per liter of water in cells and serum. The cell pH was computed by means of the Henderson-Hasselbalch equation with the pK' value of 6.04, as determined by Dill for human oxygenated blood (12). The anion equivalency of the hemoglobin at various pH values was calculated according to the equation

$$\frac{d\text{BHb}}{d\text{pH}} = 9.88$$

expressing the buffering power of oxyhemoglobin which is based on the data of Adair (13) and Maizels and Paterson (14) under the assumption of an isoionic point of 6.65 at 38°. While the precision of the value of the isoionic point is in doubt, the change with pH in the range encountered appears well established.

Results

In Table I are listed the individual data on the composition of the serum and the whole blood of seven subjects before, during, and after overbreathing. Included is a control series of observations on one of the subjects from whom five samples of blood were obtained at rest after intervals of time similar to those of experimental conditions.

In Table II are presented the mean values and their standard errors for the samples taken before and during the periods of hyperventilation. Since the values for the two samples of blood, drawn after 3 and 6 minutes of hyperventilation, respectively, did not differ significantly, except for the pH and the CO_2 tension, they were combined for the calculation of the mean values. In Table II are also given the average calculated values for the pH and the ionic equivalency of the hemoglobin in the cells. Mean values for the hemoglobin concentration in the whole blood and for the volume of packed cells are not given, since these indices differed more among the subjects than they varied during the experiment for any given individual.

In the first two rows of Table III are tabulated the means of the individual differences, between the control values on the one hand and the hyperventilation samples on the other, and the estimates of the statistical significance of these differences. In the lower two rows are listed the similarly calculated differences between the control samples and those taken

3 minutes after cessation of the overbreathing. In Table III are included the data on the changes of the volume of packed cells and of the cellular concentration of hemoglobin.

The data in Tables I to III may be briefly summarized as follows:

Serum Changes—The pH of the serum, averaging 7.47 in the control periods, rose to a mean value of 7.68 after 2 to 3 minutes of overbreathing and reached a value of 7.73 after periods of 5 to 6 minutes. It quickly fell to a value of 7.59 6 minutes after overbreathing was stopped. The pH value for the control samples appears somewhat high, probably reflecting apprehension and involuntary overbreathing of the experimental subjects.

The CO_2 content, averaging 26.3 milliequivalents per liter for the control samples, fell to a value of 20.5 milliequivalents per liter after 3 minutes of overbreathing, to remain unchanged after 'the longer periods. After 3 minutes recovery the values were still lower by about 1.5 milliequivalents per liter than at the outset. The bicarbonate concentration, 25.2 milliequivalents per liter in the beginning, fell to 19.9 milliequivalents per liter during hyperventilation.

The CO_2 tension, 36.4 mm. of Hg in the control samples, fell to 16.8 mm. after 3, and to 15.0 mm. after 6 minutes of overbreathing. It rose slowly during recovery, the values 3 minutes after stopping of the overbreathing being 10 mm. of Hg below the control level. The CO_2 tension in the control samples is somewhat lower than accepted normal values, a finding that, like the pH values, may reflect a certain degree of involuntary overbreathing of the subjects.

The serum chloride concentration, 106.3 milliequivalents per liter in the control samples, rose during the period of overbreathing to a value of 109.2, to return rapidly to the control values during recovery.

The serum sodium concentration, 137.5 milliequivalents per liter at the outset, fell to a value of 134.2 milliequivalents per liter during the period of hyperventilation and returned promptly to normal after cessation of the overbreathing.

The potassium concentration, averaging 4.3 milliequivalents per liter at the start, rose to 4.8 milliequivalents per liter during hyperventilation. It decreased promptly to the control level during recovery. This increase may possibly be due to a release of adrenalin, which is known (15) to cause a transient increase in the arterial potassium concentration.

The calcium concentration, not listed in Tables I to III, did not change significantly during the experiments from a value of 10.4 mg. per 100 cc., whether or not there were symptoms of tetany. This result is in keeping with prior reports (1).

The inorganic P fell in each experiment after 3 minutes of hyperventilation and decreased further after 6 minutes. It stayed at low levels 3 and

TABLE I

Data on Serum and Whole Blood of Seven Men before, during, and after Overbreathing

Subject No	Experimental conditions	pH in serum	CO₂ content		pCO₂	Chloride		Inorganic P in serum	Na in serum	K in serum	R.b.c.	Hb in whole blood
			Serum	Whole blood		Serum	Whole blood					
			m eq. per l.	*m eq. per l.*	*mm Hg*	*m eq per l*	*m eq per l*	*mg. per 100 cc.*	*m eq. per l*	*m eq. per l.*	*per cent*	*gm. per 100 cc.*
1	Before hyperventilation	7.44	27.1	20.2	41	107.2	82.4	2.45	139.9	4.0	43.4	15.2
	" "	7.45	25.9	20.7	38	106.4	82.8	2.28	137.2	4.5	43.4	15.1
	2.5 min. hyperventilation	7.67	22.3		19	109.2	82.0	1.95	135.1	4.5	42.1	14.8
	7.5 " "	7.75	19.6	13.8	14	109.6	81.2	1.63	134.0	4.6	43.4	15.1
	3 min. after	7.54	25.1	18.9	30	107.2	80.8	1.74	137.2	4.2	43.8	15.2
	9.5 min. after	7.42	25.4	19.1	40	108.0	82.0	1.68	137.0	4.2	43.9	15.0
2	Before hyperventilation	7.47	27.2	21.3	38	106.8	80.4	1.62	139.6	3.7	46.3	15.9
	3 min. "	7.68	21.8	15.6	18	110.4	79.2	1.27	136.4	4.5	45.1	16.0
	6 " "	7.73	20.3	15.2	15	110.4	78.8	1.20	134.6	4.6	46.1	16.2
	2 " after	7.63	24.5		23	107.2	78.0	1.20	136.2	3.8	46.9	16.0
	6 " "	7.61	26.9	18.7	27	107.6	79.6	1.27	137.9	4.0	45.6	15.8
3	Before hyperventilation	7.49	24.7	19.8	33	105.6	78.2	2.67	136.2	4.3	48.4	16.6
	" "	7.50	24.0	20.3	31	104.0	78.8	2.56	135.2	4.3	48.7	16.2
	2 min.	7.72	20.4		16	108.4	78.4	2.12	135.2	5.4	47.9	17.2
	3.5 min. hyperventilation	7.74	20.5	14.1	15	107.2	78.2	1.85		4.9	47.4	16.9
	2.5 " after	7.59	24.2	18.1	26	102.8	78.4	2.06	135.3	4.1	48.4	16.3
	5.5 " "	7.54	25.7	19.6	31	104.4	78.4	1.74	134.4	3.6	48.3	16.7
4	Before hyperventilation	7.46	26.2	21.1	38	105.6	82.0	2.67	137.5	4.5	43.2	14.9
	" "	7.48	26.4	21.3	36	106.0	82.2	2.56	137.5	4.7	41.9	14.7
	2 min. "	7.67	20.8	16.4	19	108.0	82.4	2.28	135.7	4.7	41.6	14.8
	4. " after	7.70	22.7	17.1	18	107.2	83.0	2.06	134.7	4.8	42.1	14.7
	2 " after	7.60	24.5	20.0	24	106.0	83.2	2.12	137.7	4.8	42.0	14.9
	5 " "	7.54	26.6	21.8	31	103.6	82.0	2.06	137.7	4.1	43.1	14.7

5	Before hyperventilation	7.51	24.5	20.1	31	107.2	85.8	3.35	134.8	42.8	15.5
	2.5 min. hyperventilation	7.69	18.4	13.8	15	110.8	83.4	2.95	132.9	41.0	15.0
	5 min. hyperventilation	7.71	18.8	14.5	15	111.6	83.2	2.84	132.9	42.8	15.4
	3.5 min. after	7.59	22.4	17.6	23	108.0	84.4	2.73	134.4	43.3	15.4
6	Before hyperventilation	7.46	26.0	21.4	38	106.8	83.8	1.74	134.3	40.6	13.5
	2.5 min. "	7.69	20.0	15.3	17	109.2	82.2	1.48	131.8	40.0	14.5
	5 min. "	7.75	19.9	15.0	14	108.8	82.0	1.32	131.7	40.9	14.6
	3 " after	7.59	24.3	19.4	26	106.4	81.4	1.21	133.4	41.9	14.1
7	Before hyperventilation	7.45	26.0	20.1	37	106.0	78.6	1.85	136.5	45.5	16.1
	" "	7.48	26.1	19.7	36	107.0	78.4	1.63	138.1	45.0	15.7
	3 min. "	7.67	20.1	14.8	18	109.2	79.8	1.06	134.6	44.1	16.1
	6.5 min. hyperventilation	7.75	19.4	14.6	14	108.4	79.4	0.90	135.6	44.6	15.7
	3.5 min. after	7.59	24.5	18.5	26	105.6	81.2	0.75	139.0	45.0	15.6
7	Control	7.47	27.0	20.9	38	105.2	85.0	2.39	137.5	41.0	16.1
	"	7.44	27.1	20.9	41	106.4	84.6	2.32		41.4	16.5
	"	7.50	27.5	21.3	36	106.4	85.0	2.46	138.7	42.0	15.0
	"	7.49	27.3	21.2	37	106.4	83.8	2.46		42.0	15.1
	"	7.50	27.7	21.4	35	106.4	83.6	2.32	137.3	41.6	15.3

TABLE II

Mean Values for Ionic Composition of Serum and Red Cells of Seven Men before and during Overbreathing

	pH in serum	pH in cells	HCO₃ in serum	HCO₃ in cells	pCO₂	Cl in serum	Cl in cells	Inorganic P in serum	Hb in cells	Na in serum	K in serum
			m. eq. per l	m eq per l	mm. Hg	m eq per l	m eq per l	mg. per 100 cc.	m eq per l.	m.eq. per l.	m eq. per l.
Control	7.47	7.17	25.2	12 6	36.4	106.3	52.0	2.4	27.2	137.5	4.3
	±0 01	±0.02	±0.3	±0.3	±0.7	±0.2	±1.0	±0.1		±0.6	±0.1
Over-breathing	7.71	7.34	19.9	8.2	15.9	109.2	45.0	1.8	37.3	134.2	4.8
	±0.01	±0.01	±0.3	±0.3	±0.5	±0.4	±0.6	±0.2		±0.4	±0.04

TABLE III

Mean Values for Individual Differences between Bloods before, during, and Three Minutes after Hyperventilation

	No. of differences	pH in serum	HCO₃ in serum	HCO₃ in cells	pCO₂	Cl in serum	Cl in cells	Cl in blood	Inorganic P in serum	Na in serum	K in serum	Hb in cells	Volume of packed cells
			m eq per l	m eq per l	mm Hg	m eq per l	m eq per l	m eq per l	mg. per 100 cc	m eq per l.	m eq per l.	gm. per 100 cc.	per cent
Hyperventilation vs. control	14	+0.24	−4.9	−4.7	−20 2	+2.7	−6 0	−0.7	−0.5	−2.5	+0.5	+1.0	−0.7
p*		<0.001	<0.001	<0.001	<0.001	<0.001	<0 001	0.05	<0.001	<0.001	0.01	<0.001	<0.001
Recovery vs. control	7	+0 12	−1 5	−2.2	−10.6	−0.1	−0.9	−0.6	−0.6	−0.3	−0.1	−0.1	+0.2
p*		<0.001	<0.001	0.01	<0.001	0.7	0.6	0.6	<0.001	0.7	0.4	0.6	0.4

* Per cent chance that a deviation as great or greater than that observed would arise by chance alone; any value of $p0.05$ or less usually accepted as indicating a significant difference.

6 minutes after the overbreathing stopped.   Such a fall of the inorganic P concentration was first described by Davies *et al.* (1).   It is a matter of conjecture whether this change represents an effect of alkalosis *per se* or whether it is associated with changes in the carbohydrate metabolism, perhaps in response to the release of adrenalin.   The continued fall in the concentration beyond the period of hyperventilation would favor the latter hypothesis.

Ionic Equilibrium in Red Cells—The cell pH, 7.17, in the control samples, increased to 7.34 during hyperventilation.

The CO_2 content of the cells decreased from 13.5 to 8.6 milliequivalents per liter during hyperventilation, while the bicarbonate fell from 12.6 to 8.2 milliequivalents per liter.   During recovery the increases followed a pattern similar to that observed in serum. ·

The cell chloride, 52.0 milliequivalents per liter in the beginning, diminished to 45.0 during overbreathing, returning rapidly to normal with its cessation.

The hemoglobin concentration in the cells during hyperventilation increased slightly, from about 33 gm. to 34 gm. per 100 cc.   This change corresponded in extent to the decrease in the volume of the packed cells. The ionic equivalency of the hemoglobin in the cells increased with the rise of the cell pH by 10.1 milliequivalents per liter.

Changes in Whole Blood—The CO_2 content of the whole blood decreased by about 5.5 milliequivalents per liter during hyperventilation.

The chloride remained almost unchanged during the experiments.   This may indicate that no important movement of this ion occurred to and from the blood stream.   A decrease of 0.7 milliequivalent per liter during hyperventilation, statistically barely significant, may reflect diffusion to a small extent of the chloride from the plasma into the extracellular fluid.

The volume of packed cells decreased with regularity during overbreathing, the change averaging 0.7 per cent.   It returned quickly to control values during recovery.

The hemoglobin concentration in the whole blood remained unchanged during the experiments.   This circumstance, in conjunction with the decrease in the volume of the cells, means an increase in its cellular concentration.

In Table IV is presented a balance of the changes in the ionic composition of serum and cells, based on the data in Tables I to III.   It can be seen that in the serum the concentrations of both the anions and cations decreased, their changes balancing each other closely, so that a reduction in the total electrolyte concentration resulted.   It is noteworthy that the fall in the bicarbonate content was in part accounted for by an increase in the chloride and in part by a decrease in the sodium ions.   The omission of lac-

tate, which had not been determined, does not appear to have introduced an appreciable error, probably owing to the shortness of the periods of over-breathing and to the fact that the analyses were performed on arterial blood. The changes in the serum are in keeping with the predictions of the Donnan theory, according to which they are explained on the basis of a shift of water from the cells to the serum.

<div align="center">TABLE IV</div>

<div align="center">*Changes in Ionic Balance of Serum and Cells during Hyperventilation*</div>

The differences are given in milliequivalents per liter.

	Serum	Cells
HCO_3'	−5.3	−4.4
Cl'	+2.9	−7.0
$PO_4' + PO_4''$	−0.4	
Organic phosphate*		+1.7
Protein†	+0.7	+10.1
Net change of anions	−2.1	+0.4
$Na^{\cdot}$	−3.3	
$K^{\cdot}$	+0.5	
Net change of cations		+2.0‡

* The change in the ionic equivalency of the organic acid soluble phosphates was calculated on the basis of an assumed concentration of 50 mg per 100 cc. of cells. This would correspond to a value of 35 milliequivalents per liter for the ionic equivalency of the organic phosphates in the control samples. During hyperventilation the ionic equivalency would increase by about 1.7 per cent, owing to the decreased water content of the cells, and by a further 3 per cent, owing to the increase in the cellular pH

† The change in the serum protein equivalency was calculated on the assumption of a serum concentration of 7 gm. per 100 cc. by the formula of Van Slyke *et al.* (16), on the basis of a dilution of the serum compartment of the blood by 1.8 per cent.

‡ The change in cations was calculated on the assumption of a decrease in the cell water by 1.8 per cent and of the non-diffusibility of the intracellular cations.

In the cells a decrease in the diffusible ions, chloride and bicarbonate, was closely balanced by an increase in the non-diffusible ions, primarily hemo-globin, while the change in the organic phosphates, estimated on the basis of customarily observed normal values, accounted for a small proportion only. The value listed in Table IV for the change in the cellular concentration of the cations is based on the assumption that the decreased electrolyte concentration in the serum was due to a transfer of water from the cells. Therefore, a corresponding increase in the cellular concentration of the

cations was assumed. The changes in cellular cations and anions do not balance each other as evenly as they do in the serum; however, it is doubtful that the apparently greater change in the cations than in the anions is of significance, in view of the numerous sources of error involved in the determinations and assumptions. Perhaps changes in other cellular anions, such as glutathione, which have not been taken into account, may in part even the two sides of the ledger of the ionic balance.

In Table V the assumption of a water shift between cells and serum, which has been adduced in explanation of the changes in the ionic structure, is subjected to a quantitative comparison with the corollaries of the Donnan theory. In the first two rows the changes in water content of the serum calculated on the basis of the observed changes in the concentration of the cations are compared with the theoretical water shifts calculated according to the formulation of the Donnan theory presented by Van Slyke, Wu, and McLean (6). The details of the calculations and premises involved in the calculation of the theoretical values are presented in the foot-note to Table V. Another independent test of the theory, provided by the comparison of the observed with the calculated mean change in the volume of packed cells, is listed in the next two rows. A third independent test is afforded by the comparison between the observed and the calculated changes in the cellular hemoglobin concentration. Considering the uncertainties and errors involved in the calculations and determinations, the agreement between theory and observation can be considered satisfactory.

In Table VI are tabulated the observed distribution ratios for the diffusible ions, and theoretical ratios calculated from the non-diffusible ions by means of Equation 10 of Van Slyke et al. (6) on the basis of the premises and estimates adopted for the preceding calculations. Two sets of values are listed for the theoretical ratios, one based only on the consideration of hemoglobin and the organic phosphates, and the other including an estimate of the effect of glutathione. It can be seen that the observed ratios before and during hyperventilation were about the same for chloride and for the bicarbonate, uncorrected for carbamate, while the hydrogen ion ratios were considerably lower. Correction of the bicarbonate ratio for carbamate in the cells brings its value to a point midway between the chloride and the hydrogen ratios. The theoretical ratio derived from the non-diffusible ions, particularly when glutathione was considered, approximated in its value the chloride ratio. The changes of all ionic ratios during hyperventilation were in close agreement.

It is of interest to compare these results with those of Dill et al. (8), which were based on in vitro equilibration of human venous blood. Agreement at both points of comparison, before and during hyperventilation, is very close for the distribution of chlorides and moderately good for the hydrogen ions,

while the bicarbonate ratios here reported are lower by about 0.09 for the control and by about 0.08 for the hyperventilation samples. This dis-

TABLE V

Changes in Water Distribution, Cell Volume, and Cellular Hemoglobin Concentration during Hyperventilation

		per cent
Change in water content of serum	Calculated*	−1.7
" " total ionic concentration	Observed	−1.7
" " volume of packed cells	Calculated†	−0.8
" " " " " "	Observed	−0.7
" " cellular hemoglobin concentration	Calculated	+1.7
" " " " "	Observed	+2.8

* The theoretical changes in the water content of the serum were calculated by means of Equation 23 of Van Slyke, Wu, and McLean (6) in which the parentheses

$$(H_2O)_s = (H_2O)_b \times \frac{2(B)_s - (BP)_s}{2(B)_b - (BP)_s - (BP)_c + (P)_c}$$

denote units of substance per unit of whole blood and the subscripts, s, c, b, indicate serum, cells, and whole blood, respectively. The following assumptions and substitutions were used in applying this equation. It was postulated that all factors except for $(BP)_c$ remained constant during hyperventilation. A numerical value of 0.84 gm. per cc. was assigned to $(H_2O)_b$, based on the assumed values of 0.72 gm of water per cc. of cells, and of 0.94 gm. per cc. of serum and on the observed mean value for the cell fraction of 0.444 cc. per cc. of whole blood. To $(B)_b$ was given the value of 138 milliequivalents per liter, the mean normal observed in this laboratory. For $(BP)_s$ the value of 9.5 was taken on the basis of the assumptions given in Table IV. $(BP)_c$ was calculated as the sum of the ionic equivalencies of hemoglobin and of the cell organic phosphates. For the hemoglobin, the values for ionic equivalency given in Table II, multiplied by the relative volume of the packed cells, were taken. For the ionic equivalency of the organic phosphates a constant value of 14 milliequivalents per liter of blood was adopted, based on an assumed concentration of 50 mg. of organic acid-soluble P per 100 cc. of cells, and on the determined titration curves of diphos phoglyceric acid and adenosine triphosphate (7). For $(P)_c$ a value of 9, the sum of the osmolar concentrations of hemoglobin, 5, and organic phosphates in whole blood, 4, was taken. While the precision of the various assumptions and with it the absolute values of $(H_2O)_s$ are subject to considerable doubt, this is of small importance in assessing the effect of pH on the water shift between cells and serum, since the only variable of major importance is the ionic equivalency of hemoglobin. That the various assumptions are approximately correct is indicated by the fact that the absolute value of $(H_2O)_s$ calculated by the above formula is 0.53 gm. per cc. of whole blood, compared with the initially assumed value of 0.52 gm. per cc.

† The changes in volume of packed cells were calculated by the above formula, with the hematocrit value of 44.4 per cent for the control samples.

crepancy is not easily explained by the differences in experimental arrangement.

Among the "observed" ratios, the value for the chloride generally appears the most reliable, since no assumptions or auxiliary data are required for its determination.   The variability of pK_c' with changing pH, oxygenation, and perhaps other factors affects considerably the precision of both the hydrogen and bicarbonate ratios.   Further uncertainty is introduced in the case of the latter by correction for the cellular carbamate, while the possibility of other forms of bound CO_2 cannot be ruled out entirely.   Therefore, it

TABLE VI

Observed Distribution Ratios of Diffusible Ions and Theoretical Ratios Calculated from Non-Diffusible Ions, before and during Hyperventilation

	Observed ratios				Theoretical ratios*	
	$\dfrac{[H]_s}{[H]_c}$	$\dfrac{[Cl]_c}{[Cl]_s}$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	$\dfrac{[HCO_3]_c}{[HCO_3]_s}$	Uncorrected for GSH†	Corrected for GSH‡
Control	0.51	0.64	0 65	0.56	0.69	0.66
Hyperventilation	0 43	0.56	0.56	0.48	0.62	0.58
Change	−0.08	−0.08	−0.09	−0.08	−0.07	−0.08

' The theoretical ratio was calculated according to Equation 10 of Van Slyke, Wu, and McLean (6) in which r denotes the calculated distribution ratio, and the

$$r = 1 - \frac{[BP]_c + [P_c] - [BP]_s}{2([B]_s - [BP]_s)}$$

brackets indicate concentrations per kilo of water.   The following substitutions and assumptions were made.   The water content of serum during the control periods was assumed to be 0.94 gm. per cc., and 1.7 per cent more, 0.955 gm. per cc., during hyperventilation.   The respective values for the water content of the blood cells were assumed to be 0.720 and 0.708 gm. per cc.   The bracketed expressions were calculated by means of these values on the basis of the same data and assumptions discussed in the foot-note to Table V.

† Corrected for 11 per cent carbamate (17).

‡ In this calculation the presence of glutathione in the erythrocytes was taken into account.   Its concentration was assumed to be 5 mM per kilo of cell water (18, 19), and its ionic equivalency 1.1 milliequivalents of NaOH per mM.

would appear that the close correspondence found between the theoretical distribution coefficient and the chloride ratio is of particular significance. Dill and his coworkers (8) also found the closest agreement between these two ratios.   This is of further interest, since they derived their value for the non-diffusible ions on the basis of the titration of total cellular contents of human erythrocytes, rather than on the basis of values for individual compounds in a synthetic manner, as reported here.   Each of the two methods has its drawbacks; while the determination of the total buffering power of

erythrocytes is independent of any particular knowledge or assumption with regard to the chemical constituents involved, the technique involved does not entirely prevent enzymatic breakdown of organic phosphates. The agreement of the theoretical distribution coefficients based on these two different sets of data would appear to indicate that the errors of either technique are not of major importance.

In this connection the work of Maizels and his coworkers, who investigated the importance of the non-diffusible anions other than hemoglobin in the base-binding property of erythrocytes in normal and anemic human subjects, is pertinent (14, 19). Their estimates of the ionic equivalencies for hemoglobin, organic phosphates, and glutathione are in close agreement with those presented here. However, they came to the conclusion that part of the organic phosphate must be in non-ionized form, since the sum of the anions appeared to exceed that of the cations in the erythrocytes. This conclusion hinges on the value for total cell base, 110 milliequivalents per liter of cells, adopted by them. From this value one would calculate a concentration of 152 milliequivalents of base per kilo of cell water, on the basis of an estimate of 720 gm. of water per liter of cells. This is lower by about 10 milliequivalents than the corresponding value of base in serum water. Such a finding is contrary to the postulates of the Donnan theory as well as to the findings of Maizels himself (20) and others. In our laboratory a total base concentration of 120 milliequivalents per liter of erythrocytes has been found consistently, corresponding to a value of 168 milliequivalents per kilo of cell water. If this value, which is in agreement with the Donnan theory, is applied to the data of Maizels and correction is made for carbamate, the quota of available cations is increased sufficiently to permit the assumption of full ionization of the organic phosphates in the cells in their experiments. The reason for the discrepancies in the values of the cell total base lies undoubtedly in differences in the analytical methods employed, a critical discussion of which would lead too far afield in this paper.

It is of interest to compare the changes in the electrolyte structure of the blood in hyperventilation with those found in dogs with pyloric obstruction. In the present experiments among the non-diffusible cellular anions changes in the equivalency of hemoglobin predominated, while in pyloric obstruction the increase in the diphosphoglyceric acid was of much greater importance. In the two conditions, the diffusible anions also are shifted in opposite directions. The agreement with the Donnan theory in both experimental states, characterized by widely differing electrolyte patterns, supports strongly the general applicability of the Donnan theory for the blood.

DISCUSSION

Recognition of differences in the pattern of change between arteries, capillaries, and veins may furnish the explanation of the discrepancies in

the literature concerning the rate of change and extent of elevation of serum pH during hyperventilation. The estimates of pH reported by Davies *et al.* (1) on the basis of analyses of alveolar air are in the same range as the directly determined values on arterial blood reported here and similar to those reported by Nims *et al.* (5). Analyses of venous blood have given the lowest estimates (2, 5) while intermediate values of pH have been reported on capillary blood (4). So far as the rate of change is concerned, if linearity is assumed, the pH in the present experiments increased by 0.06 to 0.09, average 0.07, unit per minute during the first 3 minutes of hyperventilation, and changed much more slowly thereafter at a rate of less than 0.02 unit per minute. Even faster initial rates were reported by Nims *et al.* (5). This initial rate is about double that recorded by Shock and Hastings (4) for capillary blood. According to Nims *et al.* (5) and unpublished experiments in this laboratory, the changes in jugular venous blood proceed at rates varying from 0.02 to 0.05 unit per minute. Such diversity in pattern may be reasonably explained on the basis of differences between the several parts of the vascular system in their relation to the lungs and the tissues. The most rapid displacement of pH would be expected in arterial blood, which is in equilibrium with the alveolar air, and is carried in thick walled vessels, which permit diffusion and exchange with the body tissues to a minor degree only. The slowest change in the ionic balance of the blood would occur in the venous blood flowing from the tissues. This change would reflect variations in the total buffering capacity of the tissues. Intermediary rates of shift might be expected in capillary blood.

The relative impermeability of the wall of the arteries to movements of water and electrolytes may also be adduced in explanation of the close agreement between the *in vivo* changes reported here and postulates of the Donnan theory which are predicated on the premise of constancy of composition of the blood. This premise would appear to be supported by the finding of unchanged concentrations of chloride and hemoglobin in the whole blood. As a consequence it would follow that the electrolyte shifts observed in arterial plasma are not representative of similar changes in the extracellular fluid. This concept is made even more plausible if one examines the opposite assumption; namely, that the changes in arterial plasma are paralleled by similar variations in the extracellular fluid. If such were the case, the increase in chloride and decrease in sodium would not be confined to plasma alone, but would occur in 30 per cent of the body fluid. Then two problems would arise: what is the origin of the chloride increment and what is the destination of the disappearing sodium? The assumption of a general shift of water between the intra- and extracellular phases of the body fluid would serve to explain the decrease in the sodium content, but would emphasize the discrepancy with respect to the chloride, and would require the additional assumption of the existence of tissue chlo-

ride stores, which would be mobilized during hyperventilation. With respect to the supposition of a water shift from tissues to extracellular fluid, the data available at present (21) suggest that the cellular fluid of muscle increases in volume during periods of prolonged hyperventilation, in contrast to the findings in blood.

It would appear from the observations of others, as well as ours, that tetany, the most frequently described symptom, usually makes its appearance after prolonged periods of hyperventilation, although it may occur occasionally as soon as 1.5 minutes after overbreathing begins. On the other hand the changes in the electroencephalogram usually begin at once and progress to a maximum within 1.5 to 2.5 minutes of hyperventilation. The type and degree of deviation of the electroencephalogram are greatly influenced by the blood sugar level (9), which does not change in a uniform manner during hyperventilation. It would appear, therefore, that the only consistent change in the electrolyte structure of arterial blood associated with the effect of overbreathing on the electroencephalogram is the rapid decrease in the CO_2 tension associated with an increase in pH.

SUMMARY

The effects of voluntary hyperventilation for periods of 2 to 3 and of 5 to 6 minutes on the electrolyte structure of arterial blood were studied on seven subjects.

1. In the serum the CO_2 tension decreased to a mean value of 15.8 mm. of Hg, while the serum pH rose to 7.71. The CO_2 content decreased from 26.3 to 20.5 milliequivalents per liter. The chloride rose from 106.3 to 109.2 milliequivalents, while the sodium decreased from 137.5 to 134.2 milliequivalents per liter. The calcium content did not change, while the potassium increased from 4.3 to 4.8 milliequivalents per liter and the inorganic P fell by 0.6 mg. per 100 cc.

2. In the red blood cells, the chloride decreased from 52.0 to 45.0 milliequivalents per liter and the total CO_2 from 13.5 to 8.6 milliequivalents per liter, while the calculated ionic equivalency of hemoglobin increased by 10.1 milliequivalents per liter. The cellular hemoglobin concentration increased by 2.8 per cent.

3. In the whole blood the CO_2 content decreased by about 5.5 milliequivalents per liter, while the chloride concentration changed little. The volume of packed cells decreased from 44.4 to 43.7 per cent.

4. The changes in the ionic distribution in serum and cells balanced each other closely. The data indicate that the electrolyte content of serum decreased by about 1.7 per cent during hyperventilation, a finding explicable

according to the Donnan theory by a shift of water from the red cells into the serum.

5. An examination of the data in the light of the Donnan theory indicates close agreement between calculated and observed water and electrolyte shifts, and in the ionic distribution ratios.

The authors wish to express their appreciation to Mrs. E. W. Brown for the statistical analysis of the data and to Mrs. Robert Schutte, Miss Ann Hobson, and Miss M. Wing for technical assistance.

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NOTES ON THE DETERMINATION AND DISTRIBUTION OF SODIUM AND POTASSIUM IN CELLS AND SERUM OF NORMAL HUMAN BLOOD*

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The distribution of bases between cells and serum of normal human blood has been studied by this laboratory over a long period of time (1, 2). Since the accuracy of the results is dependent upon the precision of the methods employed, extensive investigation of the methods for determining sodium and potassium has been included (3, 4). Since the last two publications, Consolazio and Dill (5) have reported that our method for the analysis of serum yields low values for sodium. During an exhaustive investigation we have been unable to verify this. However, it was discovered that in the determination of whole blood potassium a systematic loss had occurred in all of our previous work. A review of this investigation is here presented with the new values for the distribution of base between blood cells and serum obtained with the improved potassium technique.

Sodium Method

Consolazio and Dill reported that even in inorganic salt mixtures sodium is occluded in the neutral ferric phosphate precipitate. This trapped sodium they appeared to recover from the precipitate. Furthermore, they failed to recover added phosphate after dry ashing and concluded that all but 1 to 7 per cent of phosphate was lost by volatilization in the process of ashing.

By the use of radioactive sodium it has been possible to examine this problem by a procedure that is entirely independent of any possible errors that may reside in the chemical techniques. The radioactive sodium salts were prepared in the cyclotron of the Department of Physics, Yale University.[1] Samples of this salt were subjected to the usual procedure for ashing serum. Phosphate and iron sulfate were added in the appropriate concentrations. The resulting neutral ferric phosphate precipitates were exposed to a Geiger-Müller counter. The results of these analyses appear

in Table I. The quantity of sodium found in the precipitate did not exceed 0.5 to 1.2 per cent of the total amount in the original solution. There are no significant differences that can be related to the composition of the vessels in which ashing was carried out.

In order to recover the sodium "trapped" in the neutral iron phosphate precipitate, Consolazio and Dill (5) treated it by washing, dissolving in acid, and subsequently alkalizing with ammonia. Apparently it was assumed that phosphate was removed by this treatment. We have been unable to remove phosphorus by this procedure. Stadie and Ross (6) and Wright and Allison (7) recommend that in order to remove phosphate the iron salt must be added after the pH has been carefully adjusted to the neutral point. Subsequent addition of ammonia precipitates iron phosphate; further addition removes the remaining hydroxide. When an acid solution which contains phosphorus and iron is made alkaline with am-

TABLE I

Recovery of Radioactive Na^{24} from Neutral Ferric Phosphate Precipitate Obtained after Ashing Radioactive Sodium Chloride in Presence of Phosphate and Iron and H_2SO_4

Results are expressed in per cent of count of original sample.

Experiment No	Ashed in platinum	Ashed in porcelain	Ashed in Vycor
I		0.5*	
II	1.2*	1 1*	0 9*
III	0.8		1.0
	0.5		0.9

* The count, which was made on a solution of pooled precipitates, is expressed as the average count per single precipitate.

monia, the phosphate and iron are not completely removed. When the supernatant fluid from a solution which has been so treated is ashed, the yellow color of iron phosphate can be seen when the hot ash is removed from the muffle furnace. Upon cooling, the salt again appears white. Therefore, the presence of the iron phosphate easily escapes detection. The removal of phosphate by alkalizing an acid solution containing both iron and phosphorus is not feasible. It was, we believe, not occluded sodium, but residual phosphate which Consolazio and Dill recovered from the ferric phosphate precipitates.

It has been demonstrated that in the ashing process little or no phosphorus is lost. From inorganic solutions recoveries were complete, from serum more than 90 per cent. The low recoveries, 6 to 50 per cent reported by Consolazio and Dill (5), may not have been, as they suggested, evidence that most of the phosphate had been volatilized, but only the

failure to hydrolyze to the orthophosphate the phosphorus which had been converted to metaphosphate in the ashing process.

As the result of this renewed investigation we have found no basis for doubting the accuracy of the method used by us for the measurement of sodium in serum of whole blood. Occluded sodium in the ferric phosphate precipitate, as demonstrated by the radioactive salt, does not exceed 1 per cent, which is within the error of the method.

TABLE II

Loss of Potassium by Occlusion in Ash Not Extracted with Acid

Experiment No	Iron oxide ppt removed by centrifuging		Ppt extracted with HCl		K occluded
	Ashed in		Ashed in		
	Porcelain	Platinum	Porcelain	Platinum	
K recovered from whole blood					
	m eq	*m eq*	*m eq*	*m eq*	*m eq*
1	36 4	37 6	47 0*		9 4
2		35 6		42 0	7 4
3		33 8		40 0	6 2
4		22 2	28 2	27 0	4 8
5	29 6		41 0		11 4
6		40 8		50 6	9 8
7			43 5	44 5	
K recovered from known solution, 43 0 m eq					
8		36 8		44 0	7 2
9		42 5		42 0	0 5
10	42 8		43 7		0 9

Wet ashed in glass

Potassium Method

There is, therefore, no evidence that sodium is occluded in the iron phosphate precipitated in the analysis of either serum or whole blood by the dry ashing technique. On the other hand, it has been found that as much as 15 to 20 per cent of the potassium from whole blood may be carried down in this precipitate. This loss does not depend upon the composition of the vessels in which the process of ashing is conducted.

This loss was not detected earlier for two reasons. (1) In repeated analyses of known inorganic solutions of the approximate composition of whole blood, potassium had been recovered completely. In Table II it may be seen that recovery was satisfactory in two out of three analyses of such solutions. (2) Measurements of potassium by the present method

TABLE III

Concentrat... S... m and Potassi... S... and Cells

Subject	Dates	Volume		Water		Sodium			Potassium			Na + in cells (1)	Water of serum (2)	Distribution $\frac{(1)}{(2)}$
		Cells	Serum	Cells	Serum	Blood	Serum	Cells	Blood	Serum	Cells			
		per cent	per cent	per cent	per cent	m.eq. per l.	m.eq. per l.	m.eq. per l.	m.eq. per l.	m.eq. per l.	m.eq. per l.	m.eq. per l.	m.eq. per l.	
1942														
P. H.	Nov. 13	43.9	56.1	71.4	93.5	82.5	134.8	15.7	45.1	4.0	98.0	158.3	148.2	1.068
J. H.	" 10	47.9	52.1	71.0	93.6	79.3	135.8	17.7	48.8	4.1	97.5	162.2	150.2	1.080
A. T.	" 18	50.0	50.0	71.0	93.4	80.2	137.7	22.8	48.4	4.7	92.1	161.7	152.5	1.060
1943														
M. T.	Aug. 11	45.0	55.0	70.7	93.2	86.6	136.6	25.3	44.0	5.0	91.8	165.6	152.0	1.068
S.	Sept. 26	48.2	51.8	73.7	93.2	82.7	136.0	25.3	47.7	5.1	93.5	161.0	151.8	1.060
J.	Nov. 26	40.3		72.7	93.7	90.4	134.2	23.3	40.3	5.3	92.1	159.0	149.2	1.062
M.	" 27	42.5		72.1	93.5	91.0	141.0	23.6	42.3	3.1	95.8	165.2	154.1	1.072
K.		40.0		70.8	93.3	90.7	140.6	15.7	42.2	3.8	100.0	163.4	151.9	1.055
Average.........						85.4	137.1	21.2	44.8	4.4	95.1	162.0	151.6	1.068
Maximum......						91.0	141.0	25.3	48.8	5.3	100.0	165.6	154.9	1.088
Minimum......						79.3	134.2	15.7	40.3	3.1	91.8	158.3	148.2	1.055
Previous series, 1937														
Average.........							135.1	16.8		4.6	82.5	139.6	149.0	0.944
Maximum......							139.2	27.1		6.5	101.7	156.1	152.5	1.055
Minimum......							129.1	10.0		3.4	71.8	124.7	142.0	0.822

agreed with measurements obtained by a wet ashing procedure in which phosphate was removed by the technique of Stadie and Ross (6). This is no doubt due to the fact that the two methods are subject to the same error.

During the digestion with H_2SO_4 and HNO_3 a precipitate appears which can be dissolved only with great difficulty. This precipitate, a phosphate of iron, is removed with the phosphate and iron precipitated by the subsequent addition of ammonia. To dissolve it a high concentration of acid is required. When the solution is subsequently neutralized with ammonia, a large quantity of iron phosphate is not precipitated, but remains in the supernatant fluid. When a portion of the latter is added to uranyl-zinc acetate solution, it yields falsely high values for sodium. Since it has been found that no sodium was occluded in this ferric phosphate precipitate and since duplicate analyses for potassium repeatedly agreed, it was assumed that the precipitate could be safely disregarded. It has, however, been discovered that the precipitate does occlude potassium.

In order to recover this occluded fraction of potassium the iron salt must be converted to the chloride. This is accomplished by adding to the ash 10 drops of concentrated HCl for each cc. of blood represented by the ash. The solution is then evaporated to dryness on the steam bath. The residue is extracted with water. Consolazio, in unpublished studies, has recommended a similar procedure for the recovery of potassium from cells.

Concentrations of Potassium and Sodium in Cells and Serum of Whole Blood Measured by Revised Methods—In Table III are tabulated the concentrations of sodium and potassium per kilo of water found in a series of eight normal subjects. The values obtained for sodium in both serum and cells and for potassium in serum do not differ significantly from those found in the 1937 series (2). The slightly higher average for serum sodium in the present series may be ascribed to the inclusion in the previous series of two persons with peculiarly low sodium and in the present series of two persons with peculiarly high sodium. If these determinations are omitted, the averages of the two series are identical. The increased value for cell potassium (averaging about 13 per cent) is reflected in both the sum of sodium and potassium per kilo of cells and in the ratio of distribution of the sum of sodium and potassium per kilo of water between cells and serum. In each instance the values are higher and fall within much narrower limits.

SUMMARY

In a review of the dry ashing method previously proposed for the measurement of sodium and potassium in whole blood and serum, it has been established that no sodium is removed by the precipitation of phos-

phate.   On the other hand, in the analysis of whole blood or blood cells potassium is lost.   To avoid this source of error a modification of the procedure has been proposed.

Values for the concentrations of sodium and potassium in the cells and serum of normal human blood are presented.

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A SIMPLE METHOD OF PREPARATION OF COLLOIDAL FERROUS IRON FOR INTRAVENOUS ADMINISTRATION*

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It is frequently desirable to administer iron by vein in either experimental studies or for therapeutic purposes. When given by this route the iron is retained by the body and, as has been pointed out, can be shown to be utilized completely by the body in the production of red cell hemoglobin in iron-depleted anemic dogs.[1] Ionizable forms of iron are very poorly tolerated when given by vein.

During the course of development of methods of electroplating iron for studies of its radioactive isotopes a simple means of obtaining a high degree of dispersal of a colloidal form was encountered which may prove of value to other investigators, especially those interested in parenteral therapy. Toxicological studies have been carried out to a very limited extent. Quantities of 30 to 50 mg. of iron have been administered by vein to dogs on numerous occasions without any gross clinical symptoms or untoward reactions, and it is quite possible that much larger doses may be given safely.

Starting with a solution of ferric chloride, enough cevitamic acid is added to reduce the iron to the ferrous state (about 3 mg. of cevitamic acid to each mg. of iron present). This is then added to an equal volume of a 6 per cent solution of gelatin especially prepared for intravenous use.[2] Dilute (10 to 20 per cent) NaOH is added dropwise until color develops, at which point it has been found that the pH is in the range of 5 to 6. The resultant solution has a deep purple-brown color to transmitted light and a greenish black sheen to reflected light. It is quite stable at room temperatures. When stored in a refrigerator, the material solidifies and may be made available for injection by heating gently.

[1] Whipple, G. H., and Robscheit-Robbins, *Am. J. Med. Sc.*, **191**, 11 (1936).

[2] We are indebted to Dr. D. Tourtellotte of the Charles Knox Gelatin Company, Camden, New Jersey, for the gelatin used in this work.

THE ASSAY OF ANIMAL TISSUES FOR RESPIRATORY ENZYMES

IV. CELL STRUCTURE IN RELATION TO FATTY ACID OXIDATION*

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The "homogenate technique" is at present widely used for the study of enzymatic reactions in various types of biological material. In the original technique (1)[1] an attempt was made to eliminate cell structure and to disperse intracellular constituents so widely that endogenous respiration was eliminated by dilution, while particular phases of metabolism could be restored by the addition of appropriate substrates and cofactors. In 1942, Elliott and Libet (5) made an excellent study which led to the conclusion that "the effects of homogenization in the different media are mainly due to the tendency of isotonic salts or sugars to prevent cell cytolysis, while in hypotonic medium cytolysis occurs." They reported that isotonic preparations of brain cells compared favorably with brain slices for studies on brain metabolism, and they subsequently (6) carried out their studies with such preparations unfortified with coenzymes or with cytochrome. On the other hand, Schneider and Potter (7, 8) pursued the alternative which Elliott and Libet's work suggested and homogenized tissues in distilled water in order to make cytolysis as complete as possible. We propose to refer to this type of preparation as a "water homogenate," while that of Elliott et al. (6) may be referred to as an "isotonic homogenate." On the basis of the cytochrome c content of liver and the amount of cytochrome required to saturate the succinoxidase system (9) an objective technique for estimating the degree of cytolysis was devised (7, 8). To date no other method of obtaining a quantitative measure of cytolysis has been found; microscopic examinations cannot give quantitative data and in fact can give very erroneous impressions. According to the succinoxidase test, water homogenates of rat liver contain about 5 per cent whole cells, while isotonic preparations contain 60 to 80 per cent whole cells. As might be

[1] It may be pointed out here that the device used by Potter and Elvehjem in 1936 (1) was similar in principle to a tissue crusher described as early as 1922 (2) and subsequently modified for other purposes (3, 4). The pestles may now be obtained from the Central Scientific Company.

expected, homogenates prepared in M/30 phosphate buffer (9), which is hypotonic, yielded a figure that is intermediate, about 30 per cent whole cells. In each case, the per cent of whole cells is of course modified by the tightness of the homogenizer and the duration of the homogenization.

The importance of these considerations was recently emphasized when we attempted to devise an assay for fatty acid oxidation, following the demonstration of this reaction in homogenates (10, 11). The fatty acid oxidase activity was reported to be extremely sensitive both by Muñoz and Leloir (10) and by Lehninger (11). Since many of the findings which they reported could be interpreted in terms of cytolysis, we carried out a number of experiments in which the fatty acid oxidase activity was correlated with a "cytolysis quotient," as determined by means of the succinoxidase test, in order to determine whether cell structure is necessary for fatty acid oxidation.

<center>EXPERIMENTAL</center>

Isotonic homogenates were prepared in 0.85 per cent saline, while water homogenates were prepared in distilled water. Livers were removed from decapitated rats and chilled on cracked ice. Homogenization was carried out in cold tubes and media in a cold room, with 9 volumes of media per weight of liver. The isotonic homogenates were centrifuged at 1500 g for 10 minutes and the residue was resuspended in saline equal to one-half the original volume of the homogenate. The suspension was centrifuged down and washed twice more before suspending in one-half the original volume. The washed residue was thus equal to a 20 per cent homogenate on a volume basis. The centrifuging was identical with the procedure used by Schneider to separate nuclei from cytoplasm in water homogenates (unpublished) and was carried out in order to eliminate the endogenous respiration (Lehninger (12)). In some cases, water homogenates were prepared and made isotonic by the addition of 0.5 volume of 2.55 per cent NaCl, about 5 minutes after homogenization.

Since Lehninger (11, 12) had reported activation by adenosine triphosphate (ATP), we set up reaction mixtures in flasks with no side arms and kept them in cracked ice until the liver preparations were added, according to our previous technique for the study of oxidative phosphorylation (13), in which ATP was in the reaction mixture.

The reaction components were varied, but certain additions were constant. These included 0.3 ml. of 0.1 M Na phosphate at pH 7.5 and 0.3 ml. of 0.1 M Na malonate at pH 7.5. Other additions included 0.85 per cent NaCl, 0.01 M Na octanoate, 0.013 M Na ATP, 4×10^{-4} M cytochrome c, 0.1 M MgCl$_2$, liver preparation, and water to make 3.0 ml. The amounts added are shown in the tables.

The oxygen uptake was measured in standard Warburg apparatus at 37°. Equilibration was continued for 6 minutes and readings were taken at 5 minute intervals for 30 minutes. Center cups with 2 N NaOH and filter paper were used.

The "cytolysis quotient" was measured by determining the succinoxidase activity with and without added cytochrome c. It is an indication of the per cent of cells which have been disturbed sufficiently to lose their cytochrome c, and in order to be valid, the intact cells must contain sufficient cytochrome c to saturate the succinoxidase which they contain, and the cytochrome c of the broken cells must completely dissociate from the succinoxidase system. These requirements seem to be met in liver (7–9, and this paper), although the second requirement does not seem to hold for skeletal and cardiac muscle (7, 8). The quotient equals

$$100 \times \frac{(\text{succinoxidase with cytochrome}) - (\text{succinoxidase without cytochrome})}{\text{succinoxidase with cytochrome}}$$

Results

In preliminary tests for fatty acid oxidation with various amounts of washed residue from isotonic homogenates of rat liver, we observed vigorous oxidation (100 microliters of O_2 per 10 minutes) of octanoate in the presence of ATP when 1.0 ml. of 20 per cent washed residue was used, but no activity when 0.3 ml. of the preparation was used. There was thus a marked "dilution effect" (1). When the experiment was repeated with the NaCl content of the tissue suspension compensated, it was found that the dilution effect was due entirely to the variation in NaCl. When this was kept constant, the rate of octanoate oxidation was directly proportional to the amount of liver preparation that was added (see Table I). The data also show that cytochrome c was without effect on the rate of octanoate oxidation, while ATP was indispensable. The endogenous respiration of the preparation was zero, and the amount of oxygen uptake, corrected for the equilibration period, was close to 200 microliters. The data are thus in excellent agreement with those of Lehninger (14), who reported that octanoate was oxidized quantitatively to acetoacetic acid (theoretical oxygen uptake, 201.6 microliters) under similar conditions.

The effect of sodium chloride suggested similar data by Elliott et al. (5, 15) and raised the question of the rôle of tonicity in maintaining activity. Experiments in which the amount of magnesium chloride and sodium chloride were varied (Table II) gave strong indications that hypotonicity was detrimental to the oxidation of fatty acid.

When liver was homogenized in water, the homogenate was completely unable to oxidize octanoate (10, 12). In order to determine further the rôle of cell structure in fatty acid oxidation, aliquots of rat liver were homo-

genized in water and in saline, with the water homogenate *made isotonic* within 5 minutes after its preparation, by the addition of hypertonic saline. Aliquots of each homogenate were centrifuged and washed (see "Methods") and made up to 0.5 volume. The degree of cytolysis in each of the four preparations was estimated by determining the "cytolysis quotient." The fatty acid oxidation was measured in each of the washed preparations. The

TABLE I

Oxidation of Octanoate by Washed Isotonic Liver Homogenate

Each complete flask contained 0.3 ml. of 0.1 M Na phosphate at pH 7.5, 0.3 ml. of 0.1 M Na malonate, 0.2 ml. of 0.1 M $MgCl_2$, 0.2 ml. of 0.013 M Na adenosine triphosphate, 0.1 ml. of 4×10^{-4} M cytochrome c, 0.3 ml. of 0.01 M Na octanoate, plus 20 per cent washed isotonic liver homogenate and saline (0.85 per cent NaCl) as indicated, with sufficient water to make 3.0 ml.

Additions		Average oxygen uptake per 5 min	
Washed homogenate	Saline	Complete*	Minus cytochrome
ml	ml	microliters	microliters
0.3	0 7	17 5	18.1
0.6	0 4	35 5	35 2
1.0		53 2	54.0

* When either adenosine triphosphate or octanoate was omitted, no oxygen was taken up. In all flasks the oxygen uptake ceased rather abruptly when about 150 microliters of O_2 had been taken up. See the text.

TABLE II

Effect of Tonicity on Octanoate Oxidation

Conditions as in Table I, except 0.3 ml. of washed 20 per cent isotonic liver homogenate in all flasks, with NaCl and $MgCl_2$ varied. The amount of NaCl present includes that in the homogenate. Oxygen uptake per best 10 minutes.

0 14 M NaCl per flask	0 1 M MgCl. per flask			
	0 ml	0 2 ml	0 4 ml	0 6 ml
ml	microliters O_2	microliters O_2	microliters O_2	microliters O_2
0 3	0	12 5	30.6	40.0
0 9		35 6		
1 3	6 2	33 3	33 2	

results are shown in Table III. In the case of the washed water homogenate, no oxygen was taken up when octanoate was the substrate, and the cytolysis quotient was high, indicating almost complete cell disruption, at least to the extent that cytochrome c could diffuse away from succinoxidase. There was considerable loss in succinoxidase, due to the association of this enzyme with particles too small to be completely sedimented under these

conditions. However, considerable succinoxidase was in the form of particles which could be sedimented; these particles did not include whole cells, on the basis of the "cytolysis quotient." Furthermore, Schneider (unpublished data) has shown that cytoplasmic fractions free from nuclear nucleic acid contain granules of succinoxidase which can be sedimented under isotonic conditions. In the case of the isotonic homogenate, the "cytolysis quotient" indicated that about 75 per cent of the material was still intact enough to retain cytochrome c in association with succinoxidase, and in this preparation octanoate was vigorously oxidized. The data strongly suggest that octanoate was oxidized only by those cells which

TABLE III

Inability of Hypotonic Liver Homogenates to Oxidize Octanoate

Succinoxidase measured with a preparation equivalent to 20 mg. of fresh liver, and octanoate oxidation measured with the equivalent of 100 mg. of fresh liver. Oxygen uptake per best 10 minutes.

Preparation	Cytochrome	Succinate oxidation	Cytolysis quotient*	Octanoate oxidation
		microliters	*per cent*	*microliters*
Whole hypotonic homogenate	+	**72.4**		
	−	3.0	96.0	
Washed residue from hypotonic homogenate	+	28.8		0
	−	1.0	96.5	0
Whole isotonic homogenate	+	**71.0**		
	−	53.6	24.4	
Washed residue from isotonic homogenate	+	48.0		66.5
	−	35.6	25.8	60.6

* The "cytolysis quotient" is based upon the succinate oxidation with and without added cytochrome. Its significance is not altogether certain, but its objectivity is emphasized here by the fact that the succinate oxidation data in bold-face type are in nearly perfect agreement.

were unruptured,[2] since the granules obtained by washing the water homogenates were completely inactive.

The difficulties in assaying for fatty acid oxidation are thus apparent. Unless a cytolysis quotient is determined and the Q_{O_2} on fatty acid corrected back to 100 per cent whole cells, no quantitative data can be secured. It is difficult to prove that such a correction would be valid. However, it is of interest to see how great the Q_{O_2} on fatty acid is, even when uncorrected, since no such attempts have been made by previous investigators. From the data in Table III, assuming a water content of 70 per cent in fresh

[2] The data do not, of course, eliminate the possibility that 100 per cent of the cells were whole but had lost enough cytochrome c to make the succinoxidase activity 75 per cent of the maximum.

liver (16), the uncorrected octanoate Q_{O_2} would be 13.3 in comparison with the succinate Q_{O_2} of 72.4. However, if this figure were corrected for the degree of cytolysis and the losses due to washing, the figure would be 13.3 $\times$ 72.4 / 35.6, or 27.0, which is about one-third as large as the succinate Q_{O_2} and is 3 to 4 times as high as the Q_{O_2} of a liver slice with glucose (16). It must also be borne in mind that the observed rate is not necessarily the potential rate, since no activators other than ATP were added to the system.

The data in Table III show that active octanoate oxidation did not occur in preparations which had been washed after hypotonic treatment, and the cytolysis quotients in these inactive preparations invariably indicated loss of cell structure. Furthermore, the data in Table II show that hypotonicity was detrimental, again suggesting the need for cell structure. But if the enzyme system *per se* were inhibited by hypotonicity, this data would not be conclusive, and if the system included soluble proteins, these would surely be washed away from the laked cells, while they would probably not escape from the whole cells. The use of unwashed water homogenates is complicated by the endogenous respiration, and also by the presence of calcium ions. Experiments in which washed isotonic homogenates were frozen and thawed showed that the cells were laked, on the basis of the cytolysis quotients, and concomitantly the ability to oxidize octanoate was lost. However, this is not conclusive, since freezing might damage the octanoate system inasmuch as it has been shown to damage the oxidative phosphorylation previously studied (13).

What appears to be unimpeachable evidence was obtained by carefully washing aliquots of an isotonic homogenate, and then suspending one of the final residues in distilled water to lake the washed cells, while the other aliquot was suspended in saline as before. The laked cells were added to Warburg flasks containing enough sodium chloride to make the final reaction mixture optimum for both laked cells and whole cells. The laked preparations gave results which were nearly always inferior to the washed cells, both in rate and duration of oxygen uptake, and the data were not satisfactory until potassium ions were included in the final reaction mixture. This was done in order to compensate for losses which may have occurred during the washing in isotonic sodium chloride, to compensate for the decrease in K^+ concentration due to dispersal of cell contents upon lysis (17), and because Lehninger's reaction mixture (12) had included K^+ ions. Data from a single experiment are given in Table IV to show the effect of both K^+ and cytochrome c in both whole cell and laked cell preparations which have been washed, with cytolysis quotients determined on the basis of the succinoxidase system. This experiment has been repeated a number of times in this exact pattern, with the same excellent

agreement between the rates of octanoate oxidation in laked preparations as compared with the whole cell preparations, *when both cytochrome c and K+ ions are present* in the reaction mixture. It is noteworthy that cytochrome c is shown to stimulate octanoate oxidation when laked cells are used but not when whole cells are used, thus explaining the data in Table I. The data in Table IV are apparently the first demonstration of the participation of cytochrome c in the ATP-activated oxidation of octanoate. We have encountered a number of special situations, to be reported elsewhere, in which washed cells will oxidize octanoate but laked aliquots are inactive,

TABLE IV

Octanoate Oxidation in Washed Rat Liver Cells Following Lysis

Succinate oxidation was measured as previously described (7), with enzyme preparations equivalent to 20 mg of fresh liver. Octanoate oxidation was measured with reaction mixtures which contained phosphate, malonate, octanoate, adenosine triphosphate, and cytochrome, as in Table I, plus 0.2 ml. of 0.2 M MgCl$_2$ and water to make 3 0 ml in the final volume The washed cells were added as 0.5 ml. of a 20 per cent suspension in saline, and the laked cells were added as 1.0 ml. of a 10 per cent suspension in distilled water Sodium chloride was added as 0.2 ml. of 0.42 M solution, and K$^+$ ions were added as 0 1 ml of 0.42 M of KCl substituted for an equivalent amount of NaCl Oxygen uptake reported on the basis of the best two 5 minute readings.

Washed cells	K$^+$	Cytochrome	Succinate oxidation	Cytolysis quotient	Octanoate oxidation
			microliters	*per cent*	*microliters*
Whole	−	−	24.2	44	37.8
	−	+	43.5		43.5
	+	−			42.7
	+	+			48.3
Laked	−	−	0	100	8.1
	−	+	46.6		31.0
	+	−			8.7
	+	+			49.2

with or without added K$^+$ ions. In other cases, the laked cells oxidize octanoate nearly as well in the absence of added K$^+$ as in its presence. In general, there must be some activity with Na$^+$ ions only, in order for K$^+$ ions to exert an effect. It seems not unlikely that the action of potassium is indirect, and since Lehninger has reported that DPN[3] is a component of the system (12), it may be a deficient component in some of the inactive preparations encountered. With the technique for demonstrating octanoate oxidation in laked cell preparations thoroughly established, it was possible to test some of the questions raised in the preceding paragraph.

[1] Diphosphopyridine nucleotide.

It was found that the enzyme system was sensitive to hypotonic media and that data analogous to that in Table II were obtained with the laked washed cell preparations, which were in addition somewhat more sensitive to hypertonic media than were the whole cell preparations. Furthermore, the laked cell preparations lost their activity upon freezing (in liquid air), showing that this type of experiment could not be used to test the rôle of cell structure in octanoate oxidation. The conclusion that fatty acid oxidation does not require cell structure is thus based upon the data in Table IV.

Microscopic examination of the preparations was carried out with about 9 volumes of Loeffler's methylene blue (alcoholic) to 1 volume of preparation. Nuclei are stained dark blue, nucleoli very dark blue, and cytoplasmic granules light blue. In unwashed homogenates almost no conclusions could be drawn, due to the masking of whole cells by adhering cytoplasmic granules. Even in the washed isotonic preparations this effect was noticeable. However, in the latter it was easier to discern chains of liver cells, rather uniform in size, about 3 to 4 times as long as they were broad, though the cell membranes did not stand out as smooth boundaries. On the other hand, some whole cells could be seen even in water homogenates. The microscopic examination provided no reliable guide as to the extent of cytolysis.

DISCUSSION

Whether Leloir and Muñoz or Lehninger have obtained oxidation of higher fatty acids independent of cell structure cannot be stated definitely at this time, since they have not determined "cytolysis quotients" by the succinoxidase test or by some equally objective measurement. All of the published data seem to emphasize the importance of avoiding conditions which would logically be expected to produce cytolysis, and in some of the data the omission of cytochrome c from the reaction mixture, or the lack of an increased oxygen uptake when cytochrome was added, strongly suggests that whole cells were being used. The data presented in Table IV, on the other hand, seem to provide clear cut proof that octanoate oxidation, at least, is possible in the absence of cell structure. The chief point to be emphasized is that the cells must not be laked prior to the washing procedure in order to obtain activity in the present reaction mixture.

One of the striking new developments in Lehninger's work is the ATP activation of fatty acid oxidation. We have completely confirmed this observation, and although our experimental techniques deliberately deviated considerably from Lehninger's, we adhered quite closely to the principles which his work established and used most of the components of the reaction mixture at the concentrations he found to be optimum. Thus it

seems likely that, whether or not the previous work was actually done with cell-free preparations, fatty acid oxidation would have been obtained if the washed preparations had been laked before they were tested.

The ATP activation of octanoate oxidation in preparations of "whole" (*i.e.*, unlaked according to the cytolysis quotient) cells was rather unexpected, since phosphorylated coenzymes and intermediates have generally been found to penetrate whole cells very poorly in comparison with the non-phosphorylated derivatives; *cf.*, for example, the comparison of thiamine with cocarboxylase in brain cell suspensions (18), in which thiamine was superior to cocarboxylase in minced tissue while the reverse was true in more finely ground ("broken cell") suspensions. It is unclear whether the latter would have been classified as whole or laked by the succinoxidase technique. More recently, isotonic homogenates have given marked responses to the addition of phosphorylated cofactors and intermediates (19), although the preparations were not washed and therefore must also have contained some broken cells. Nevertheless the water homogenates seemed superior since, when properly fortified, they gave somewhat greater activity than the isotonic homogenates, possibly "because there is no permeability barrier between the [added] coenzymes and enzymes in disrupted cells" (19). It seems possible that the permeability of the washed cells has been altered by the washing to the extent that the ATP molecule can get in, while the much larger cytochrome molecule cannot get out.

The significance of the present study is not simply whether fatty acids can be oxidized in the absence of cell structure, particularly since the essential means of securing this result were fairly well established by the work of Leloir and Muñoz and of Lehninger. However, it may be anticipated that other metabolic reactions which have hitherto required intact cells may be attacked with the object of demonstrating the reaction in cell-free extracts. The present study may serve to show how the homogenate technique may be employed to gain this end and to help establish the conclusion.

Although assays for fatty acid oxidase are not yet possible on a strictly quantitative direct basis, considerable information can be obtained by means of the washed preparations if satisfactory succinoxidase assays with and without cytochrome *c* can be obtained on the original homogenate as well as on the washed preparation.

SUMMARY

1. The variation in the extent of cell rupture in "isotonic," "hypotonic," and "water" homogenates is emphasized, and the inadequacy of microscopic examination is pointed out.

2. An objective test, which measures the loss of cytochrome *c* from cells

in liver homogenates, is utilized to determine a "cytolysis quotient" in the various types of homogenates.

3. The activation of fatty acid (C_8) oxidation by adenosine triphosphate in the presence of washed cells prepared from isotonic rat liver homogenates has been confirmed.

4. Washed rat liver cells which were laked with distilled water gave fatty acid oxidase activity equal to that of isotonic control suspensions, when the reaction media contained K^+ ions and cytochrome c in addition to the components used with the suspensions.

5. Water homogenates made isotonic and washed with isotonic saline gave residues which would oxidize succinate but not octanoate under the conditions which permitted oxidation in the laked washed cell preparation.

6. Due to the above considerations, the assay of fatty acid oxidase activity cannot be made by direct means as yet.

The author is happy to acknowledge the helpful criticism of Dr. Albert Lehninger, Department of Surgery, University of Chicago, to whom this manuscript was sent before its final revision.

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THE LACTOBACILLUS CASEI FACTORS IN THE NUTRITION OF THE CHICK

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Stokstad and Manning (1) demonstrated the existence of an unidentified growth essential for chicks which was termed factor U. Snell and Peterson (2) indicated the existence of a growth factor of unknown composition for *Lactobacillus casei*. Because of its properties, the substance was designated the "norit eluate factor." Hutchings *et al.* (3) presented evidence showing that the "norit eluate factor" was concerned in chick nutrition, thus suggesting a relationship between factor U and the "norit eluate factor."

Since that time several crystalline products have been isolated. Pfiffner *et al.* (4) isolated a substance from liver which was essential for *Lactobacillus casei* and was active in promoting growth and hematopoiesis in the chick. This material was termed vitamin B_c. Stokstad (5) isolated a crystalline compound from liver and one from yeast. The liver compound was active for *L. casei*, *Streptococcus faecalis* R, and the chick. The yeast compound had the same activity as the material isolated from liver when assayed with *L. casei*, but was only one-half as active as the liver compound when assayed with *Streptococcus faecalis* R. These compounds were subsequently referred to as the liver and yeast *L. casei* factors. The synthesis of the liver *L. casei* factor was recently announced by Angier *et al.* (6).

The isolation of another form of the *Lactobacillus casei* factor was described by Hutchings *et al.* (7). This compound which was obtained from a fermentation product was 85 per cent as active as the liver *L. casei* factor when assayed with *L. casei*, but only 6 per cent as active when assayed with *Streptococcus faecalis* R.

In a later communication, Hutchings *et al.* (8) presented quantitative data on the activity of the fermentation compound for the chick. The fermentation *Lactobacillus casei* factor was approximately as active as the liver *L. casei* factor in promoting growth and hemoglobin formation in the chick. It was suggested that the activity of a compound for *L. casei* would be a more general indication of its growth-promoting and anemia-preventing properties for the chick than would be the *Streptococcus faecalis* R activity of the compound.

In this communication further experiments with the fermentation

Lactobacillus casei factor are described. Data are also presented for the requirement of the chick for the synthetic liver *Lactobacillus casei* factor.

<div align="center">EXPERIMENTAL</div>

The composition of our basal diet is shown in Table I. Day-old New Hampshire red chicks were given this diet and water *ad libitum* and were housed in heated batteries. The *Lactobacillus casei* factor preparations were made up at frequent intervals by dissolving the factor in 0.05 N sodium hydroxide at a concentration of 1 mg. per ml. Fresh diets were made up weekly and various amounts of the *Lactobacillus casei* factor were added directly to the basal diet.

The stock mash was a commercial chick starter fortified with 3 per cent each of dried whole liver, dried brewers' yeast, and cerophyl.

The chicks were weighed twice weekly and all experiments continued for 4 weeks. Hemoglobin determinations were made by the method of Evelyn (9).

<div align="center">*Results*</div>

The results with the fermentation *Lactobacillus casei* factor are presented in Table II. Each series represents an experiment, done at a different time with a separate lot of chicks. Series 1 and 2 show the effect of several levels of the fermentation *Lactobacillus casei* factor. Also shown (Series 3) is the effect of the addition to the diet of several sulfonamides that are known to inhibit intestinal bacteria. The sulfonamides showed no effect except a slight growth depression with sulfaguanidine, which was also manifested on the fortified stock diet. It is to be noted that our diet contained 5 mg. of *p*-aminobenzoic acid per kilo.

Low levels, 0.16 to 0.48 mg. of the fermentation *Lactobacillus casei* factor per kilo of diet, produced pronounced responses in growth and hemoglobin formation. The type of anemia which was encountered was macrocytic and hyperchromic. These results are in agreement with those of Campbell *et al.* (10). Maximum growth was obtained with approximately 1.1 mg. per kilo of diet with no further increase when the levels were increased up to 4.0 mg. per kilo. The growth obtained equals that on the fortified stock diet.

The experiments with the synthetic liver *Lactobacillus casei* factor are tabulated in Table III. Although very marked responses are evident at low levels, maximum growth and hemoglobin values are obtained between 0.5 to 1.0 mg. of the synthetic compound per kilo of diet.

The absorption spectra of the fermentation *Lactobacillus casei* factor and the synthetic liver compound are similar, differing only in their extinction coefficients: $E_{1\ cm}^{1\%}$. at 365 mμ in 0.1 N NaOH 130 for fermentation *L. casei*

factor, and 199.0 for synthetic liver *L. casei* factor. If we assume that the chromophoric groups of the two factors are the same, the ratio of the $E_{1\,cm.}^{1\%}$ values at a given wave-length is a ratio of the molecular weights of the two compounds. On this comparative basis the fermentation *L. casei* factor should be 65.4 per cent as active as the synthetic liver *L. casei* compound. The results presented demonstrate that the ratio of activity of the two compounds is of this general order. The biological variation of the

TABLE I

Composition of Basal Diet

Ingredient		Ingredient	
	per cent		*mg. per cent*
Cerelose*	53.0	Biotin	0.03
Alcohol-extracted casein	22.0	Riboflavin	0.5
Salt mixture	4.3	Inositol	100.0
Calcium gluconate	3.0	*p*-Aminobenzoic acid	5.0
Gelatin	8.0	Vitamin E	5.0
Ruffex†	4.0	" K	0.2
Soy bean oil	5.0		*units*
Cholic acid	0.25	" A	3500.0
Cystine	0.45	" D	200.0
	mg. per cent	Oral dosing, 3 drops once	
Choline chloride	200.0	a wk.,	
Calcium pantothenate	3.0	Vitamin A	7000.0
Nicotinamide	3.0	Delsterol	400.0
Pyridoxine	0.5		*.mg.*
Thiamine hydrochloride	0.3	Vitamin E	5.0
		" K, in 0.1 cc.	
		corn oil	0.005

* Glucose monohydrate.

† A purified cellulose containing 70 per cent α-cellulose and 30 per cent other celluloses. The Fisher Scientific Company, Pittsburgh, Pennsylvania.

chick experiments makes it difficult to assess the comparative activity more accurately.

DISCUSSION

Elvehjem and coworkers (11, 12) and Norris and associates (13) have failed to find any correlation between microbiological assay and the growth-promoting and hemoglobin-forming properties of various concentrates when assayed with chicks. An explanation of their difficulties was suggested by the fact that various naturally occurring compounds do exist, and have varying potencies for *Lactobacillus casei* and *Streptococcus faecalis* R.

Hence, any conclusive comparative data based on microbiological and chick assay of impure concentrates would be difficult to obtain. The magnitude of these difficulties became apparent when Pfiffner *et al.* (14)

TABLE II

Requirement of Chick for Fermentation Lactobacillus casei Factor

Series No.	No. of chicks	Supplement	Average weight and No. alive* at 28 days	Average hemoglobin at 28 days
		per kilo	*gm.*	*gm. per cent*
1	11	None	95 (4)	4.8
	11	0.16 mg. *L. casei* factor	156 (6)	5.4
	11	0.32 " " " "	159 (9)	6.0
	11	0.48 " " " "	214 (11)	7.1
	11	0.64 " " " "	215 (10)	7.0
	11	0.80 " " " "	243 (10)	7.7
	11	1.12 " " " "	266 (11)	7.2
	11	1.50 " " " "	271 (11)	7.7
	11	4.00 " " " "	260 (11)	7.4
2	10	None	130 (1)	3.9
	12	0.48 mg. *L. casei* factor	237 (11)	7.5
	12	0.64 " " " "	283 (12)	8.3
	12	0.80 " " " "	244 (12)	8.6
	12	0.96 " " " "	272 (12)	8.3
	12	1.12 " " " "	291 (11)	8.0
	12	1.28 " " " "	274 (12)	8.4
	10	2.40 " " " "	279 (10)	8.4
	10	Fortified stock mash	309 (10)	7.8
3	10	None	105 (2)	
	10	0.80 mg. *L. casei* factor	278 (10)	7.8
	10	4.00 " " " "	302 (10)	7.1
	10	0.80 " " " " + 0.7% sulfa-suxidine	293 (9)	7.6
	10	0.80 mg. *L. casei* factor + 0.7% sulfa-guanidine	254 (10)	7.8
	10	0.80 mg. *L. casei* factor + 0.7% carboxy-sulfathiazole	292 (10)	8.2
	9	Fortified stock mash	305 (9)	7.48
	10	" " " + 0.7% sulfa-guanidine	281 (9)	

* Figures in parentheses.

isolated a crystalline conjugate from yeast that was available to chicks, but which was inactive for bacteria prior to enzyme digestion. The evidence of numerous investigators (15–18) conclusively shows that such micro-biologically inactive conjugates are of wide occurrence in natural products.

Briggs *et al.* (19), using the fermentation *Lactobacillus casei* factor of Hutchings *et al.* (7), attributed vitamin "B$_{10}$" and "B$_{11}$" activity to the compound. However, the fact that the amounts necessary for growth and hemoglobin formation were higher than the amount of *Lactobacillus casei* factor present in the crude concentrates, as was determined by microbiological assay, led these workers to the conclusion that considerably more active compounds existed. Scott *et al.* (20), using the same compound, attributed growth-promoting and hemoglobin-forming properties to the compound, but only in the presence of β-pyracin.

TABLE III

Effect of Synthetic Liver Lactobacillus casei Factor on Growth and Hemoglobin Formation in Chicks

Series No. (10 chicks each)	Supplement	Average weight and No. alive* at 28 days	Average hemoglobin at 28 days
	per kilo	*gm.*	*gm. per cent*
4	None	65 (1)	5.96
	0.2 mg. synthetic *L. casei* factor	255 (8)	6.48
	0.3 " " " " "	293 (8)	7.15
	0.4 " " " " "	333 (10)	7.88
	0.5 " " " " "	284 (10)	8.21
	1.0 " " " " "	343 (10)	8.59
5	None	(0)	
	0.3 mg. synthetic *L. casei* factor	277 (9)	7.53
	0.5 " " " " "	327 (9)	8.62
	0.7 " " " " "	346 (10)	7.58
	1.0 " " " " "	380 (10)	8.68
	1.5 " " " " "	366 (10)	8.07
	5.0 " " " " "	387 (10)	7.80

Figures in parentheses.

The data presented above indicate that on a molar basis the fermentation *Lactobacillus casei* factor is as active in promoting growth and hemoglobin formation in the chick as is the synthetic compound, which is identical with the naturally occurring liver *Lactobacillus casei* factor. This is the most active form of the compound for either microorganisms or chicks. The fermentation compound was active under the same experimental conditions as the synthetic compound. The addition of β-pyracin was not necessary to elicit a response.

In view of the apparent discrepancy between the activity of vitamin B$_c$, as reported by Pfiffner and associates, and the activity of the fer-

mentation *Lactobacillus casei* factor, as reported by Scott and coworkers, on chicks, Daniel *et al.* (21) have advanced *in vitro* experiments purported to reconcile these differences.

In a study of the conversion of the fermentation *Lactobacillus casei* factor to a compound more active for *Streptococcus faecalis* R by an enzyme present in chick liver tissue, these investigators found that β-pyracin was concerned in the reaction. Their explanation was that either β-pyracin was conjugated with the *L. casei* factor to form "folic acid" or the presence of β-pyracin facilitated the conversion. Attention is directed to the paper of Day *et al.* (22) in which the enzyme present in rat liver that is capable of hydrolyzing microbiologically inactive conjugates was efficacious in converting the fermentation *L. casei* factor into a compound with increased activity for *Streptococcus faecalis* R. The addition of β-pyracin was not necessary for this reaction. Furthermore, previously published data (7) of the absorption spectra of the various *L. casei* factors would indicate that the fermentation compound is a higher molecular weight compound than the liver *L. casei* factor. It would thus appear likely that the enzymatic activation was of a degradative nature.

The results presented substantiate our previous conclusion that the fermentation *Lactobacillus casei* factor is active in promoting growth and hemoglobin formation in the chick. Evidence presented in this paper indicates that the fermentation compound is as active on a molar basis as the synthetic liver *Lactobacillus casei* factor.

Smaller amounts of either compound are necessary for hemoglobin formation than for maximum growth.

The amounts of the fermentation *Lactobacillus casei* factor and the synthetic compound necessary to give growth equivalent to that of a fortified stock mash are 1.1 mg. per kilo of diet and 0.5 to 1.0 mg. per kilo of diet respectively.

SUMMARY

The fermentation *Lactobacillus casei* factor and the synthetic liver *Lactobacillus casei* factor are active in promoting growth and hemoglobin formation in the chick. On a molar basis the two compounds are approximately equally active. On our basal diet the two compounds are active under identical conditions. The addition of β-pyracin is not necessary for growth or hemoglobin formation.

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THE REPLACEMENT OF VITAMIN A₁ BY VITAMIN A₂ IN THE RETINA OF THE RAT*

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It is now well established that vision in dim light is due to the photo-chemical decomposition of "visual purple," a pigment present in the rods of the retina (1). Visual purple is believed to be a conjugated protein in which vitamin A or one of its derivatives is a prosthetic group (2). As might be expected, the spectral sensitivity·curve of the eye parallels the absorption spectrum of visual purple with a maximum at 500 mμ, except for a slight shift toward the red due mainly to light absorption of the intraocular media.

In 1937, Wald (3) found that certain fresh water fish have a visual purple system which differs from that found in man and most other animals. The absorption of this pigment occurs at about 522 mμ, and the entire absorption spectrum is shifted somewhat toward longer wave-lengths. It is reasonable to assume that in dim light these fish are comparatively more sensitive to red light and less sensitive to blue than animals with the normal type of visual purple. To avoid confusion, the visual purple of humans and salt water fish was named rhodopsin, while the visual purple of fresh water fish was named porphyropsin.

At about this same time, other investigators (4, 5) found that the livers of these fresh water fish contained, instead of vitamin A, a closely related substance having an absorption maximum in the ultraviolet at about 350 mμ instead of 328 mμ and that the antimony trichloride reaction product had an absorption maximum at 693 mμ instead of 620 mμ. This material was named vitamin A₂, and shortly thereafter Wald (6) showed this substance to be the prosthetic group in porphyropsin, as vitamin A is in rhodopsin. It has subsequently been found (7, 8) that vitamin A₂ has biological activity for rats and occasionally occurs in small amounts in the livers of animals that eat fresh water fish (9).

It would be of considerable interest to find out whether the spectral

response curve of the human retina can be shifted toward the red region by the substitution of porphyropsin for rhodopsin in the retina. As the initial step in such a study, we investigated the possible replacement of vitamin A_1 by vitamin A_2 in the retina, liver, and blood of the albino rat.

Procedure

132 male and female rats were placed at weaning (24 days old) in individual cages and given the "vitamin A test diet" (10). After 9 weeks on this diet, the usual vitamin A deficiency symptoms developed and the colony was divided into two groups. One group of 63 vitamin A-deficient rats was examined immediately; a group of 76 normal rats was examined simultaneously for comparison. The remainder of the colony was placed on a daily supplementation of 100 "units" of vitamin A_2[1] given orally by dropper in Mazola. The vitamin A_2 was extracted from the livers, pyloric ceca, gastrointestinal tracts, and body fat of wall-eyed pike (*Stizostedion vitreum*). These extracts all had an ultraviolet absorption maximum at 352 mμ, with a subsidiary peak at 286 mμ. The antimony trichloride products all showed a single absorption maximum at 695 mμ.

Groups of six to eight rats were taken at intervals of 3, 6, and 12 weeks and examined to see what changes had occurred in the vitamin A_1 and A_2 levels in the retina, blood, and liver. After 12 weeks on vitamin A_2 feeding, a group of forty-eight rats was examined.

At the time of sacrifice, the rats were placed overnight in a dark room and killed by decapitation. A minimum of illumination was furnished by a Kodak Series No. 2 Safelite. The blood was collected and allowed to clot and the serum taken. A representative number of livers were removed, and the pooled sample weighed and placed under 95 per cent ethyl alcohol. The eyes were removed promptly and placed in normal saline solution. They were transferred to a 4 per cent alum solution for a period of about 2 hours. The lenses were removed, and the retinas dissected out and placed in a phosphate buffer solution (pH 6.8), centrifuged, and washed with the same solution repeatedly. The washed retinas were ground with sand under 4 per cent sodium glycocholate solution and the volume brought up to about 15 ml. After standing for an hour, the mixture was centrifuged and the supernatant liquid poured off.

[1] Since there is no standard unit of vitamin A_2, we have adopted, *pro tem*, the expedient of employing an arbitrary physicochemical or spectral unit. This is determined by using the same conversion factors as are used in this laboratory for calculating units of vitamin A_1 from its extinction coefficient at 328 mμ in the ultraviolet or the extinction coefficient at 620 mμ for its antimony trichloride product. Thus the potencies in units of vitamin A_2 were calculated by multiplying the extinction coefficient at 352 mμ by ●000 and, for the antimony trichloride product, by multiplying the extinction coefficient at 695 mμ by 750.

The transmission spectra of the retinal extracts were determined on a Hardy spectrophotometer against a blank sample of the 4 per cent sodium glycocholate solution. The small amount of light needed to give the transmission spectra produced no bleaching of the visual purple. Subsequently, the retinal extracts were exposed to room light until no further fading occurred at 500 mμ, and curves for the bleached samples were obtained. Since these extracts all had considerable general absorption in the 400 to 500 mμ region, the curve of the bleached sample was subtracted from the original in order to obtain a better curve of the visual purple itself. ·It must be noted, however, that this procedure would yield a true spectrum of the visual purple only if it is assumed that no other products absorbing in the same region are produced at this time. Since it is known (11) that some such products are formed, the curve obtained by subtraction is not a true curve of the visual purple. However, for the purposes of this experiment, the difference is not significant.

Retinene was extracted with benzene from the bleached visual purple solutions, and its antimony trichloride spectrum recorded.

The blood sera and the livers were extracted in the usual manner and spectra of the antimony trichloride colors obtained.

Results

Amount and Character of Visual Purple—As can be seen from Fig. 1, the normal rats had a visual purple curve with a maximum at 500 mμ, showing that rhodopsin was present.[2] The vitamin A-deficient rats examined at the same time also showed the presence of rhodopsin but in a much smaller amount. In contrast, the large group of rats which had been on vitamin A$_2$ supplementation for 12 weeks gave a visual purple curve with an absorption maximum at 520 mμ. Since the absorption maximum of porphyropsin occurs at 522 $\pm$ 2 mμ, this shift of the spectrum indicated that the visual purple of the vitamin A$_2$-fed rats had been changed to porphyropsin to the extent of about 80 per cent or more.

The primary question with which this investigation is concerned is whether or not an animal normally utilizing only vitamin A$_1$ in its retinal pigment can produce the typical vitamin A$_2$-containing visual purple. Fig. 1 gives a clear cut answer to this question. On prolonged feeding of vitamin A$_2$, the rat has almost entirely replaced vitamin A$_1$ by vitamin A$_2$ in the visual purple of its retina.

Retinene—The bleached visual purple extracts were extracted with

[2] The retinas from thirty-three frogs were examined to test our experimental procedures. An excellent rhodopsin curve was obtained. The frog retinas were found to contain about twice as much visual purple as those of the normal albino rat. The livers contained 165 units per gm. of vitamin A$_1$.

benzene and examined for retinene, which gives a characteristic antimony trichloride blue color with a maximum at 664 mμ (2). Definite evidence of retinene was found in the extract from the visual purple of normal rats. No retinine could be detected in the benzene extract from the bleached visual purple of rats which had been fed vitamin A₂ for 12 weeks. However, in the latter case, the presence of retinene was not expected, since, according to Wald (6), bleached porphyropsin yields a different substance, retinene₂. This compound gives an antimony trichloride product having an absorption maximum at 706 mμ. Unfortunately, this wave-length was beyond the limit of the spectrophotometer used.

Vitamin A in Retina—The amount of vitamin A₁ in the bleached retinas of two small groups of normal rats was found to be 0.64 and 0.87 units per
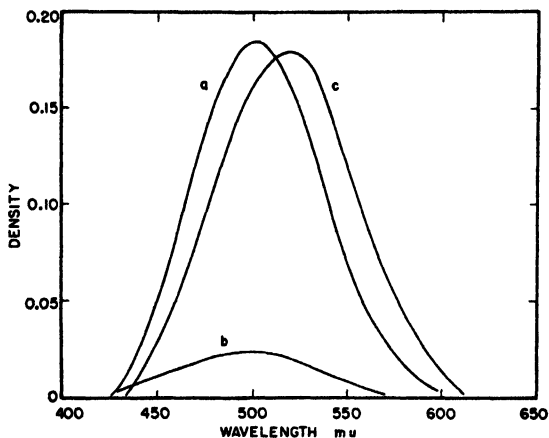
Fig. 1. Absorption spectra of the visual purple extracts from normal rats (Curve a), vitamin A-deficient rats (Curve b), and previously depleted rats fed a supplement of 100 units daily of vitamin A₂ for a period of 12 weeks (Curve c).

rat. A comparative measurement on the retinas of a small group of vitamin A-deficient rats showed only one-fourth to one-third as much vitamin A (0.23 unit per rat) as is normally present. After 3 weeks of feeding 100 units of vitamin A₂ daily, the retinas of a small group showed 0.83 unit of vitamin A₁ per rat. No evidence of vitamin A₂ was observed. After 6 weeks of vitamin A₂ supplementation, the retinas from another small group showed 0.75 unit of vitamin A₁ per rat, with the appearance of just a trace of vitamin A₂. It appears that the retinas pick up vitamin A₂ extremely slowly and tend to maintain normal levels of vitamin A₁. Since all of the available retinas were needed for the visual purple estimation on the rats fed vitamin A₂ for 12 weeks, no vitamin A determinations were made on the bleached retinas of these animals.

Vitamin A₂ in Liver—The livers of normal rats from our stock colony were found to contain 125 to 135 units of vitamin A_1 per gm. As may be seen from Fig. 2, where the spectra of the antimony trichloride products are given, the extract of normal rat livers shows a single absorption maximum at 620 mμ. The curve for the liver extracts from vitamin A-deficient animals also has its absorption maximum at the same wave-length, but the peak is markedly depressed and corresponds to about 8 per cent (10.5 units per gm.) as much vitamin A_1 as was found in the normals.

The large group of vitamin A-deficient rats placed on 100 units of vitamin
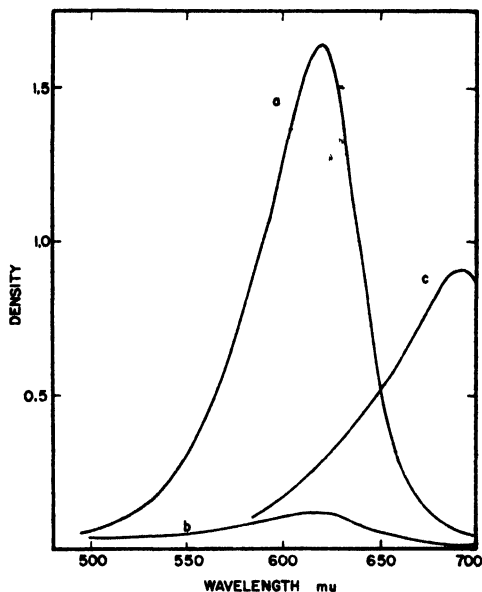
FIG. 2 Absorption spectra of the antimony trichloride reaction products from the livers of normal rats (Curve *a*), vitamin A-deficient rats (Curve *b*), and depleted rats fed a supplement of 100 units daily of vitamin A_2 for a period of 12 weeks (Curve *c*).

A_2 daily resumed growth promptly, and vitamin A deficiency symptoms disappeared. Groups of six rats each were sacrificed after receiving vitamin A_2 supplementation for 3, 6, and 12 weeks. The livers from the rats after 3 weeks contained only vitamin A_2 to the extent of about 52 units per gm. There was no indication of a tendency to increased deposition of vitamin A_2 on continued feeding. Thus, the livers of rats after 6 and 12 weeks contained 36 and 40 units of vitamin A_2 per gm., respectively. The livers from the rats of the large group on vitamin A_2 supplementation for 12 weeks (Fig. 2) showed only vitamin A_2 (antimony trichloride absorption maximum

at 695 mμ).   At this time, the livers contained 76 units of vitamin A₂ per gm.   The prompt rise in the vitamin A₂ level of the rat livers to values of 36 to 76 units per gm. and the maintenance at this level during continued feeding of vitamin A₂ are evidence of a systemic balance which maintains a constancy of vitamin A₂ in the liver at a given level of intake.   Such a phenomenon has been previously noted by Lewis et al. (12), who report that when vitamin A-deficient rats were placed on an intake of 100 units of vitamin A₁ daily the livers establish a store of approximately 113 units per gm.

Several points emerge from these data.   In vitamin A-deficient rats fed vitamin A₂, the vitamin A₂ appeared promptly in the liver and established a definite storage level.   In this respect, vitamin A₂ apparently follows a pattern of systemic behavior similar to that of vitamin A₁.   Only vitamin A₂ was found in the liver even after as short an interval as 3 weeks of vitamin A₂ supplementation.   In the liver, vitamin A₂ appears to be a suitable biological replacement for vitamin A₁.

Vitamin A₂ in Blood—The blood serum from two large groups of normal rats was found to contain 1.0 and 0.7 units of vitamin A₁ per ml.   The curve for the antimony trichloride product (Fig. 3) of the normal rat blood extract showed a maximum at 620 mμ.   The curve of the vitamin A-deficient rat blood extract showed a single maximum at 620 mμ, but the height of the absorption curve was greatly reduced, as compared to that of the normals. The blood of these rats contained only 0.2 unit of vitamin A₁ per ml. or about one-fourth to one-fifth as much vitamin A₁ as is normally present.

The blood serum from a group of six rats was examined after 6 weeks on vitamin A₂ supplementation, and it was found that vitamin A₂ had appeared in the blood (0.2 unit per ml.), although vitamin A₁ still predominated (0.3 unit per ml.).[3]   This relationship was reversed after 12 weeks of vitamin A₂ supplementation, at which time 0.2 unit per ml. of vitamin A₁ and 0.3 unit per ml. of vitamin A₂ were found in the serum.   An anomalous finding is recorded in the values for the blood serum of a group of six rats chosen at random after 12 weeks on vitamin A₂ supplementation.   In this serum only vitamin A₁ was present in the amount of 0.8 unit per ml.   No reason for this anomaly is known.

In contrast to the rapid uptake of vitamin A₂ by the liver, the blood seemed to retain vitamin A₁ tenaciously.   It would appear that 100 units of vitamin A₂ have been transported daily by the blood stream, deposited in the liver and other tissues, metabolized, and perhaps excreted for as long a

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[3] Jensen et al. (8) have found from a study of carefully prepared concentrates that the antimony trichloride product of vitamin A₁ has absorption at 695 mμ equal to about 5 per cent of its absorption at 620 mμ, while the antimony trichloride product of vitamin A₂ contributes absorption at 620 mμ equal to about 33 per cent of its absorption at 695 mμ.   The relative amounts of vitamins A₁ and A₂ in mixtures were calculated on this basis.

period as 6 weeks without significantly altering the vitamin A_1 level in the blood as compared to that of vitamin A-deficient animals. The tenacity of retention of vitamin A_1 is further demonstrated by the amount of vitamin A_1 found in the blood after 12 weeks of vitamin A_2 feeding. The total amount of combined vitamin A_1 and vitamin A_2 in the blood serum after 12 weeks is nearly up to the normal level of vitamin A_1 in rat blood. However, it is apparent that prolonged periods of vitamin A_2 feeding would be necessary to replace vitamin A_1 completely, if indeed this is possible. It may be that small amounts of vitamin A_1 are stored in many tissues of the body and
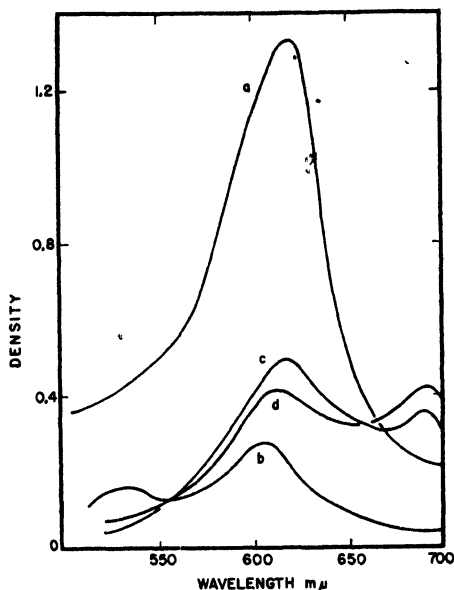
Fig. 3. Absorption spectra of the antimony trichloride reaction products of the extracts from blood serum of normal rats (Curve a), vitamin A-deficient rats (Curve b), depleted rats fed a supplement of 100 units daily of vitamin A_2 for 6 weeks (Curve c), and rats fed the vitamin A_2 supplement for 12 weeks (Curve d).

that upon feeding vitamin A_2 these stores are gradually replaced. The vitamin A_1 thus released perhaps finds its way into the blood stream and is responsible for its continued appearance there. However, vitamin A_2 appears in the blood stream in a reasonably constant amount and seems, over a protracted period of vitamin A_2 feeding, to be gradually replacing the vitamin A_1.

Functional Replacement of Vitamin A_1 by Vitamin A_2—One of the important generalities which emerges from this study is the apparent ability of vitamin A_2 to replace vitamin A_1 in general body functions. The vitamin

A-deficient rats without supplementation of vitamin A presumably would have died. When 100 units daily of vitamin A_2 were administered to each rat, all symptoms of vitamin A deficiency rapidly disappeared; growth was immediately resumed and the xerophthalmia healed. The coats became healthy looking and the rats were lively, ate well, and were in excellent condition. The livers promptly took up stores of vitamin A_2 and vitamin A_2 appeared in the blood and in the visual purple. The survival and growth on vitamin A_2 supplementation and especially the production of porphyropsin in the retinas indicate the usefulness of vitamin A_2 in those body functions in which vitamin A_1 is considered necessary.

Vitamin A_2 Reproduction—After 6 weeks on vitamin A_2 supplementation, the rats were mated; eight groups of two female rats were placed in separate cages, with one male for each group. Only half of the females became pregnant, but this percentage is not extraordinarily low in first matings. Only one of the females raised its young, although all that were pregnant delivered litters. In general, the pups were not very strong and varied markedly in weight in each litter. The pups that died had stomachs well filled with milk, showing that the failure was not in the milk supply. The one surviving litter was placed on 33 units of vitamin A_2 daily at weaning, and, during the latter part of the lactation period, the mother was put back on the usual dosage of 100 units daily. The pups had grown well at the time of sacrifice, having body weights of 56 to 80 gm. Unfortunately, the extracts of blood, livers, and retinas of the pups were cloudy and no satisfactory measurements of the antimony trichloride products of these tissues could be obtained. The loss of such a high percentage of the litters is unusual and may reflect some fault in the ability of vitamin A_2 to replace vitamin A_1. Further evidence of such a fault may be found in the wide variation in the size of the pups. The value of vitamin A_2 in reproduction and the survival of young rats is open to further experimentation.

Sex Differences in Response to Vitamin A_2—In comparison of the growth curves of the rats following vitamin A_2 supplementation, a difference was noted in the growth responses of male and female rats. For example, after 3 weeks on 100 units of vitamin A_2 the two male rats in one small group had increased about 35 gm. in body weight, whereas the four female rats had increased only 10 gm. After 6 weeks on vitamin A_2 feeding, the male rat in another small group had gained 100 gm. in body weight, whereas the five female rats had increased only 35 gm. on the average. In the large group during the 7 weeks for which weight records on vitamin A_2 supplementation are available, the twenty-eight male rats showed an average increase of 50 gm. in body weight, while the sixteen female rats showed an average increase of only 27 gm. This sex difference is also supported by evidence gained from an experiment in which two male and two female rats on a normal

intake of vitamin A_1 were fed 10,000 units daily of vitamin A_2 for 2 weeks. At the end of this time, the livers were examined separately.   The livers of the male rats contained 39 and 68 units of vitamin A_1 per gm. and 171 and 254 units of vitamin A_2 per gm., respectively.   The livers of the female rats contained 112 and 133 units of vitamin A_1 per gm. and 103 and 130 units of vitamin A_2 per gm., respectively.   Thus, the proportion of vitamin A_2 to vitamin A_1 was much higher in the livers of the male rats than in those of the females.   However, evidence for sex difference in utilization of vitamin A_2 by males and females is insufficient to be regarded as conclusive.

It should be noted here that, aside from the difference in growth response of the male and female animals, neither sex shows the weight gain that would be predicted for a daily supplementation of 100 units of vitamin A on the assumption that vitamin A_2 is equal in potency to vitamin A_1.   This is in line with the observations of both Gillam *et al.* (7) and Jensen *et al.* (8), who found that the growth response to higher levels of vitamin A_2 feeding was not as great as would be predicted from the response at low levels.   The latter workers also noted that very high doses of vitamin A_2 (10,000 units per day) were much more toxic than equivalent doses of vitamin A_1. This phenomenon may reflect some difference in biological response to vitamin A_2 or may be caused by some contaminant in the fresh water fish liver extract.

SUMMARY

1. Vitamin A_2 can be incorporated into the visual purple of the albino rat, an animal normally utilizing only vitamin A_1 in this retinal pigment.

2. Upon administration of 100 "units" of vitamin A_2 daily, the liver of the albino rat promptly develops and maintains a store of vitamin A_2.

3. Upon continued feeding of vitamin A_2, the blood of the albino rat slowly increases in vitamin A_2 content while tenaciously holding to the vitamin A_1 available.

4. Vitamin A_2 appears to replace vitamin A_1 successfully in many important body functions of the rat.

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THE SYNTHESIS OF POTENTIAL ANTIMALARIALS

DERIVATIVES OF PANTOYLTAURINE*

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The general hypothesis as to the mode of action of chemotherapeutic agents, which has been formulated by Fildes, Woods, McIlwain, and others (2), offers a rational and useful guide to the design of new drugs. Thus, bacteriostasis is pictured as caused by the blocking of reactions essential to growth by an inhibiting substance which has a structure similar to that of one of the normal enzymes or metabolites essential to the growth of the organism.

There is some indication that such a mechanism may also be involved in the case of protozoa, since it is known that certain of the sulfonamides are plasmodicidal *in vivo*. To extend this approach to *Plasmodium* by attempting to block essential metabolites or enzymes other than those involved in the action of the sulfonamides has been made difficult by the lack of knowledge of the essential metabolic requirements of the parasite.

An important lead to this approach has been furnished by Trager (3), who showed that the survival *in vitro* of *Plasmodium lophurae* is favored by the presence of calcium pantothenate. Although the evidence is indirect, Trager's results indicate that pantothenic acid is the only growth factor of known chemical structure thus far demonstrated for any species of *Plasmodium*.

The hypothesis of Fildes *et al.* has been tested experimentally by the design and preparation of several new growth inhibitors for bacteria (2). In the case of pantothenic acid, Snell (4) was the first to report the preparation of a salt of *dl*-pantoyltaurine which inhibited the growth of certain bacteria *in vitro* and he showed that the inhibition was reversed by panto-

thenic acid. In the same year Kuhn, Wieland, and Möller (5) prepared solutions of the d and l modifications of pantoyltaurine and they reported that the d form was 32 times more active than the l form as a growth inhibitor for certain bacteria. Independently, McIlwain, Barnett, and Robinson (6) prepared and tested as bacterial inhibitors not only dl-pantoyltaurine but also dl-pantoyltaurylamide and a number of other analogues of pantothenic acid.

In the light of Trager's evidence that pantothenic acid is an essential growth factor for *Plasmodium lophurae* and of the work of Snell, McIlwain, and Kuhn, showing the existence of several compounds known to interfere with pantothenic acid metabolism, at least with respect to bacteria, the advisability of testing pantothenic acid inhibitors for antimalarial activity becomes evident.

The work reported here describes the preparation, in as pure a form as possible, of the optically active d-pantoyltaurine (I) and several of its derivatives, II, III, IV, V, VI, and VII, primarily for testing as antimalarials and incidentally for testing with a variety of other pathogens.

$$HO—CH_2—C(CH_3)_2—CH(OH)—CO—NH—CH_2—CH_2—SO_2—R$$

I. R = —ONa

II. R = —NH₂

III. R = —NH—

IV. R = —NH—

V. R = —NH—

VI. R = —NH—CH

VII R = —NH—CH₂—(CH₂)₁₆—CH₃
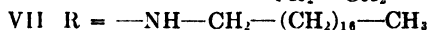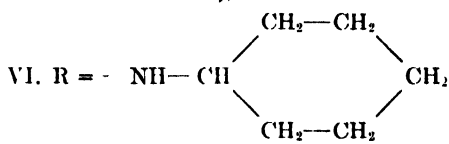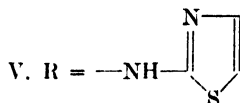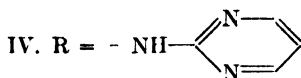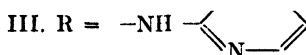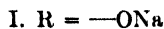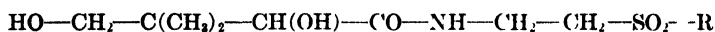
A report of the results obtained with compounds I to VII in tests on avian malaria will be reported elsewhere.[1] It may be stated, however, that d-pantoyltaurylamide (II) showed definite activity *in vivo* in the suppression of parasites under certain test conditions.

<center>EXPERIMENTAL</center>

d-Pantoyltaurine (Sodium Salt) (I), (SN 327)[1]—This compound was prepared in a manner similar to that employed by Snell (4) for the preparation of the optically inactive compound. The preparation of solutions of the d and l modifications has been reported by Kuhn *et al.* (5).

To 36.4 gm. of thoroughly dried l-pantolactone were added 41.2 gm. of

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[1] The Survey number, designated SN, identifies a drug in the records of the Survey of Antimalarial Drugs. The antimalarial activities of the compounds to which Survey numbers have been assigned will be tabulated in a forthcoming monograph.

the dried sodium salt of taurine and the mixture heated between 115–120° for 4.5 hours with occasional stirring. Bubbles of gas appeared to evolve during the reaction. The melt was poured into absolute ethanol and, upon standing, formed a gel. The product was extracted with 700 ml. of absolute ethanol, the ethanol concentrated *in vacuo* to 100 ml., and 600 ml. of acetone added. A powder precipitated which, after standing overnight at 0°, was filtered, washed with acetone, and dried over sulfuric acid *in vacuo*. There were obtained 46.5 gm. (90 per cent on the basis of lactone consumed) of a white deliquescent powder melting with effervescence at 100–110°. $[\alpha]_D^{23} = +23.4°$ (27.2 mg. in 1.93 ml. of water).

$C_8H_{16}O_6NSNa$ Calculated.   C 34.7, H 5.8, N 5.1, Na 8.3
          Found   " 34.5, " 6.1, " 4.8, " 8.4

d-Pantoyltaurylamide (*II*), (*SN 3279*)—Taurylamide hydrochloride, prepared by the method of Miller, Sprague, Kissinger, and McBurney (7), was converted to the free base as described by Barnett and Robinson (8). The oil thus obtained crystallized to a solid melting at 90–100°; because it is hygroscopic, it was used without further purification. Taurylamide (19.3 gm.) was heated with *l*-pantolactone (21.0 gm.) at 100–110° for 3 hours (see Barnett and Robinson's (8) procedure for the preparation of the optically inactive compound). The product (39 gm.) was a hygroscopic viscous gum which could not be induced to crystallize. A sample was purified for analysis by repeated precipitations from ethanol with isopropyl ether; after prolonged drying over phosphorus pentoxide *in vacuo*, the *d*-pantoyltaurylamide consisted of a colorless glassy solid. $[\alpha]_D^{23} = +19.1°$ (52 mg. in 2.0 ml. of water).

$C_8H_{18}O_5N_2S$.   Calculated, N 11.0; found,[2] N 10.7

β-Phthalimidoethanesulfonyl-2-aminopyridine—Since the condensation of the acid chloride and 2-aminopyridine, whether carried out in pyridine or in water with sodium carbonate, always resulted in the hydrolysis of the sulfonyl chloride, the following conditions were employed: To 25 gm. of 2-aminopyridine in the minimum amount of benzene were added 35 gm. of β-phthalimidoethanesulfonyl chloride (7) in benzene. The resulting solution was refluxed for 1 hour and allowed to cool. The precipitate, which formed during the reaction, was filtered off, stirred with dilute sodium bicarbonate solution, filtered, washed with water, and dried over sulfuric acid *in vacuo*. For purification, this product was refluxed with 150 ml. of methanol, allowed to cool, and filtered. There were thus obtained 33 gm. of β-phthalimidoethanesulfonyl-2-aminopyridine, colorless prisms, m.p. 213–215°.

[2] A semimicro-Kjeldahl determination was carried out by Mr. C. T. Redemann.

$C_{15}H_{13}O_4N_3S$.   Calculated.   C 54.4, H 4.0, N 12.7
                   Found.   " 54.7, " 4.0, " 12.4

Tauryl-2-aminopyridine—To 23 gm. of the above β-phthalimidoethane-sulfonyl-2-aminopyridine suspended in 200 ml. of hot ethanol were added 11.7 ml. of 42 per cent hydrazine hydrate (9), and the mixture was refluxed on the water bath. During 15 minutes, the solid had dissolved, and a precipitate had begun to form. The mixture was refluxed for 1 hour, cooled, and filtered. The intermediate thus obtained was dissolved in 1 liter of hot water and treated with 13.5 ml. of concentrated hydrochloric acid. When the solution had cooled, the phthalhydrazide was filtered off, and the filtrate evaporated to dryness. The solid thus obtained, after several crystallizations from ethanol, proved to be a mixture of the mono- and di-hydrochlorides of taurylaminopyridine. Although they could never be completely separated, it was found that the monohydrochloride melted at about 165°, while the dihydrochloride had a melting point of about 190°. The hydrochlorides were each treated with the required amount of sodium bicarbonate solution. On evaporation of the aqueous solutions and crystallization of the residues from absolute ethanol, the same compound was obtained from both hydrochlorides as colorless clusters of platelets, m.p. 140–141°.

$C_7H_{11}O_2N_3S$.   Calculated.   C 41.8, H 5.5, N 20.9
                   Found.   " 41.9, " 5.8, " 20.8

d-N²-(Pantoyltauryl)-2-aminopyridine (*III*), (*SN 3280*)—To 4.5 gm. of tauryl-2-aminopyridine were added 3 gm. of *l*-pantolactone, and the mixture was heated at 115–120° for 5 hours. After standing overnight in a desiccator over sulfuric acid, a product was obtained which solidified upon dissolving in acetone and pouring the solution into a large volume of dry ether. The material which precipitated was filtered under anhydrous conditions and dried over phosphorus pentoxide *in vacuo*. The 5.5 gm. of semicrystalline material thus obtained were hygroscopic and gradually became dark and gummy on exposure in the air. The best samples decomposed so rapidly that satisfactory analyses were not obtained. They had a melting point of about 53°. $[\alpha]_D^{23} = +18.5°$ (62 mg. in 2.0 ml. of water).

β-Phthalimidoethanesulfonyl-2-aminopyrimidine—To 17.2 gm. of 2-amino-pyrimidine suspended in 50 ml. of dry pyridine were added slowly, with shaking, 46.5 gm. of β-phthalimidoethanesulfonyl chloride. The solid gradually dissolved, and, after an hour, a flocculent precipitate began to form. The suspension was stirred vigorously overnight and then poured into 1 liter of water. The resulting suspension was neutralized with sodium bicarbonate and filtered. After drying, the product was obtained as 34.3 gm. (58 per cent) of a brown powder melting at 245–250°. After several

recrystallizations from glacial acetic acid a sample was obtained as colorless prisms, m.p. 245–247°.

$C_{14}H_{12}N_4O_4S$. Calculated, C 50.6, H 3.6; found, C 50.5, H 3.7

Tauryl-2-aminopyrimidine Hydrochloride—The hydrolysis of the phthalimido compound was carried out with hydrazine hydrate and hydrochloric acid solution, as described for the preparation of tauryl-2-aminopyridine. The hydrochloride was obtained in 75 per cent yield as colorless prisms from dilute ethanol, m.p. 215–216°.

$C_6H_{11}N_4O_2SCl$. Calculated. C 30.2, H 4.6, N 23.5
Found.   " 30.2, " 4.6, " 23.9

Tauryl-2-aminopyrimidine Hydrate—On neutralization of an aqueous solution of the hydrochloride with sodium bicarbonate, a precipitate was obtained which after filtering and drying consisted of flat hexagonal prisms. A sample of this material, when heated, softened above 140° and melted at 151° with decomposition.

$C_6H_{10}N_4O_2S \cdot H_2O$. Calculated. C 32.7, H 5.5, N 25.4
Found.   " 33.0, " 5.2, " 25.3

Tauryl-2-aminopyrimidine—On heating at 100°, the hydrate slowly decomposed. About 85 per cent of the theoretical amount of water was removed by drying at 106–110° for 4 hours in a vacuum over phosphorus pentoxide. The compound thus obtained regained the water on standing in the air. It has not been obtained in pure form because of its instability, and the crude material was used for the next reaction.

d-N²-(Pantoyltauryl)-2-aminopyrimidine (IV), (SN 7293)—Finely powdered tauryl-2-aminopyrimidine hydrate (1.1 gm.) was dried at 92° under a high vacuum for 18 hours. To the resulting solid was added 0.9 gm. of *l*-pantolactone, and the mixture heated at 97–98° for 6 hours and at 115° for an additional 30 minutes. A clear melt was obtained. The temperature was lowered to 100° and the heating continued for 8 hours. During this latter period, some crystallization was observed. The reaction mixture was dissolved in hot ethanol, filtered, and allowed to stand overnight. The supernatant solution was decanted from a small amount of hygroscopic amorphous solid and treated with isopropyl ether. After standing for some time, small clumps of crystals were deposited. After filtering and drying, the product (0.45 gm.) was recrystallized from absolute ethanol and colorless clusters of crystals, m.p. 177–178.5°, were obtained. $[\alpha]_D^{23} = +23.6°$ (21.9 mg. in 1.99 ml. of water).

$C_{12}H_{20}O_5N_4S$. Calculated. C 43.4, H 6.1, N 16.9
Found.   " 43.7, " 6.1, " 16.9

β-Phthalimidoethanesulfonyl-2-aminothiazole—To 19 gm. of 2-aminothiazole in the minimum amount of benzene were added 25 gm. of the β-phthalimidoethanesulfonyl chloride in benzene, and the solution was refluxed for 1 hour.   After cooling, the solid which had precipitated was filtered off and allowed to stand overnight in sodium bicarbonate solution.   It was then filtered, washed with water, dried over sulfuric acid, and crystallized from glacial acetic acid.   There were obtained 27 gm. of a product with a melting point of 227–228°.

$$C_{13}H_{11}O_4N_3S_2. \quad \text{Calculated,} \quad C \ 46.3, \ H \ 3.3; \ \text{found,} \ C \ 46.6, \ H \ 3.3$$

The attempted hydrolysis of this compound with hydrazine hydrate and dilute hydrochloric acid was accompanied by a large evolution of hydrogen sulfide and led to the production of a dark oil, from which no identifiable compound could be obtained.

Tauryl-2-aminothiazole Hydrochloride—A solution of 76 gm. of β-phthalimidoethanesulfonyl-2-aminothiazole in 1500 ml. of 92 per cent ethanol containing 54 gm. of sodium hydroxide was heated at 60–65° for 24 hours. It was acidified with 10 N ethanolic hydrogen chloride and, after standing in the cold for several hours, filtered from the sodium chloride.   The filtrate was evaporated to dryness under reduced pressure, and the residue dissolved in acetone and filtered to remove the unchanged starting material.   The oil thus obtained was extracted with 170 ml. of water in portions and decanted from a small amount of insoluble oil (starting material and phthalic acid). Evaporation of the solution gave 15 gm. of crude tauryl-2-aminothiazole hydrochloride, which was recrystallized from glacial acetic acid to give 13.3 gm. of rosettes of needles, m.p. 187–190.5°.   An analytical sample melted at 193–195°.   From the reaction there were recovered 29 gm. of starting material.

$$C_5H_{10}O_2N_3S_2Cl. \quad \text{Calculated.} \quad C \ 24.6, \ H \ 4.1, \ N \ 17.2$$
$$\text{Found.} \quad \text{``} \ 24.8, \text{``} \ 3.8, \text{``} \ 17.2$$

N^2-*(Pantoyltauryl)-2-aminothiazole* (*V*), (*SN 9667*)—To 1.7 gm. of tauryl-2-aminothiazole hydrochloride in 2 ml. of warm water was added 0.6 gm. of sodium bicarbonate.   The resulting brown solution was dried *in vacuo* over sulfuric acid and finally over phosphorus pentoxide.   To the above mixture of tauryl-2-aminothiazole and sodium chloride was added 0.91 gm. of *l*-pantolactone, and the mixture heated at 80–90° for 2 hours.   The product was extracted with 10 ml. of hot absolute ethanol, filtered, and the solvent removed under reduced pressure.   The residual brown oil was washed repeatedly with cold acetone and finally dried *in vacuo* over phosphorus pentoxide, whereupon it solidified to a light brown powder.   The product was soluble in water, but, on standing in solution, decomposed with evolu-

tion of hydrogen sulfide. It was also unstable in moist air, but remained unchanged over long periods when kept over phosphorus pentoxide. No significant rotation could be observed.

$C_{11}H_{19}O_5N_3S_2$. Calculated. C 39.2, H 5.7, N 12.5
Found. " 39.5, " 5.8, " 12.8

β-Phthalimidoethanesulfonylaminocyclohexane—To 13.7 gm. of β-phthal-imidoethanesulfonyl chloride dissolved in 250 ml. of acetone were added 10.5 gm. of cyclohexylamine. The solution was refluxed for 1 hour and then poured into a large volume of water. The solid was recovered by filtration and recrystallized from ethanol to give 15 gm. of long colorless needles, m.p. 152–153°.

$C_{16}N_{20}O_4N_2S$ Calculated. C 57.1, H 6.0, N 8.3
Found " 57.5, " 6.2, " 8.0

Taurylaminocyclohexane–The phthalimido compound was hydrolyzed with hydrazine hydrate and hydrochloric acid, as described for the prepara-tion of tauryl-2-aminopyridine. The taurylaminocyclohexane was obtained in quantitative yield by neutralization of the hygroscopic hydrochloride followed by evaporation of the aqueous solution and extraction of the resi-due with ethanol. On recrystallization of the crude product from ethanol, there was obtained a colorless product, m.p. 92–93°.

d-Pantoyltaurylaminocyclohexane (*VI*), (*SN 3281*)– To 6.2 gm. of tauryl-aminocyclohexane were added 4 gm. of *l*-pantolactone, and the mixture heated at 120° for 4 hours. The resulting oil was solidified by dissolving it in acetone and pouring the solution into dry ether, but it could not be in-duced to crystallize. There were thus obtained 9 gm. of the crude prod-uct, which was used for testing. A portion of this product was dissolved in a small amount of water, and this solution, after standing for several days, slowly deposited colorless crystals, which were filtered off and re-crystallized several times from water plus a few drops of ethanol, to give colorless needles, m.p. 125–126°. $[\alpha]_D^{23} = +2.7°$ (67 mg. in 2.0 ml. of water).

$C_{14}H_{28}O_5N_2S$ Calculated. C 50.0, H 8.4, N 8.3
Found. " 50.5, " 8.3, " 8.3

β-Phthalimidoethanesulfonylaminooctadecane—To a solution of 20 gm. of β-phthalimidoethanesulfonyl chloride dissolved in the minimum amount of benzene was added a solution of 41 gm. of octadecylamine in benzene. The solution was refluxed for 1 hour and then evaporated to dryness under reduced pressure. The residue (about 50 gm.) was recrystallized from ethanol to give 33 gm of colorless plates, m.p. 109–109.5°.

$C_{28}H_{46}O_4N_2S$. Calculated. C 66.4, H 9.2, N 5.5
                  Found.   " 66.4, " 9.3, " 5.7

The alcoholic mother liquors were evaporated almost to dryness and poured into a large amount of ether, whereupon the octadecylamine hydrochloride precipitated in a fairly pure form, m.p. 155–160°.

Taurylaminooctadecane—The hydrolysis of the phthalimido compound was carried out with hydrazine hydrate, as described for the preparation of tauryl-2-aminopyridine. The combined precipitate of phthalhydrazide and taurylaminooctadecane hydrochloride was centrifuged down, and, after removal of the excess hydrochloric acid by several washings with water followed by recovery of the precipitate by centrifuging, the solids were treated with two successive portions of normal sodium hydroxide solution. The product from this treatment was filtered, washed thoroughly with water, and dried over sulfuric acid. The resulting solid was twice crystallized from ethanol to give 16 gm. of colorless needles, m.p. 90–91°. The basic filtrate and washings could be acidified to recover the phthalhydrazide.

$C_{20}H_{44}O_2N_2S$. Calculated, N 7.2; found, N 7.2

d-Pantoyltaurylaminooctadecane (*VII*), (*SN 3282*)—To 19.8 gm. of the above taurylaminooctadecane were added 7.5 gm. of *l*-pantolactone, and the mixture heated at 100° for 2 hours. The resulting solid was recrystallized from ethanol to give 23 gm. of colorless prisms, m.p. 98–100°. $[\alpha]_D^{23} = +51.0°$ (46 mg. in 5.0 ml. of chloroform).

$C_{26}H_{54}O_5N_2S$. Calculated. C 61.7, H 10.7, N 5.5
                  Found.   " 61.7, " 10.8, " 5.6

Attempted Preparation of Pantoyltauryl-9-aminoanthracene (*SN 5923*)—Although the instability of certain of the intermediates and of the final product has precluded satisfactory analyses, we wish to record the results obtained. 9-Nitroanthracene (10) was prepared and reduced to 9-aminoanthracene (11, 12) with stannous chloride. The compound had a melting point of 148–151° but decomposed slowly on standing in a closed vessel. The anthramine was condensed with β-phthalimidoethanesulfonyl chloride in pyridine to give β-phthalimidoethanesulfonyl-9-aminoanthracene (?) as a light buff-colored solid which did not melt below 300°. The phthalimido compound was hydrolyzed with hydrazine hydrate and the resulting tauryl-9-aminoanthracene hydrochloride (?) had a melting point of 205–206° (with decomposition) when recrystallized from ethanol. The hydrochloride was neutralized with sodium bicarbonate and the free base recrystallized from ethanol to give light buff-colored crystals, m.p. 174–175°. The free base gradually decomposed on standing and was therefore immediately condensed with *l*-pantolactone by heating for 3 hours at 120°. The

product was crystallized from cellosolve and light yellow crystals of pantoyl-tauryl-9-aminoanthracene (?), m.p. 219–220°, were obtained. Recrystal-lization from cellosolve or prolonged standing at room temperature was accompanied by decomposition, as evidenced by darkening. The analyses gave results too variable to be of significance.

The authors wish to thank Dr. William Trager of the Rockefeller Insti-tute, Princeton, New Jersey, for his encouragement and suggestions and Dr. Carl Niemann and Dr. Edwin Buchman of this Institute for their advice and technical aid. We are indebted to Merck and Company, Inc., for a generous gift of *l*-pantolactone and 2-aminothiazole, to the American Cyanamid Company for a gift of 2-aminopyrimidine, and to Armour and Company for a gift of octadecylamine. The microanalyses, except where indicated, were performed by Dr. Gertrude Oppenheimer of this Institute.

SUMMARY

The preparation is described of a series of optically active derivatives of pantoyltaurine, in as pure a form as possible, for testing for antimalarial activity.

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STUDIES ON CHOLINE ACETYLASE

II. THE FORMATION OF ACETYLCHOLINE IN THE NERVE AXON

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The high concentration of cholinesterase in the axon and its exclusive localization at the neuronal surface are one of the essential facts supporting the concept that the release of acetylcholine is directly associated with the nerve action potential; *i.e.*, with the conduction of the nerve impulse (1, 2). The enzyme found in the axon is an esterase specific for acetylcholine (3). The presence of a specific and highly active enzymatic mechanism for the removal of acetylcholine in the axon indicates that the ester is metabolized there at a high rate and suggests the possibility that acetylcholine may be formed in the axon as well as at the synapse.

Acetylcholine is, as shown by Nachmansohn and Machado, synthesized by an enzyme system, choline acetylase, which can be extracted from brain but not from other organs (4). Several properties of the enzyme have been described in previous communications (5–7). It appeared of interest to test whether or not choline acetylase occurs in the axon.

The evidence that energy-rich phosphate bonds are the primary energy source during the recovery period following the action potential and may account for the electric energy released is based on observations on the electric organ (8). These organs are an accumulation of end-plates. Their action potential is therefore more comparable to the end-plate potential than to the axon potential. When it was concluded that energy-rich phosphate bonds should account for the resynthesis of the primary compound split during nerve activity, *i.e.* acetylcholine, evidence for such a mechanism was offered with solutions prepared from brain. The presence of choline acetylase in the brain could, however, still be attributed to the presence of nerve endings. No such objection is possible if the same enzyme mechanism is found in the peripheral fiber free of endings. It appears probable that the physicochemical mechanism of the end-plate potential is identical with that of the axon potential. Evidence that the complex energy-using enzyme system required for the formation of acetylcholine is present in the axon would be new additional support for the assumption that the rôle of the ester is not limited to the nerve ending but is of equal importance in the axon.

The peripheral fiber offers moreover a favorable material to correlate

directly acetylcholine metabolism and function.   In the degenerating fiber it is easy to follow the decrease of enzyme activity and to compare it with the loss of conductivity.   •

The presence of choline acetylase in the peripheral fiber has been reported in a preliminary note (9).   In this paper the full material is presented.

Methods

The extract was prepared in the same way as was described previously (5, 7).   Six to seven rabbits were used for each experiment.   The sciatic nerve was thoroughly ground in a homogenizer with phosphate buffer and silica.   After centrifugation at 3000 R.P.M. for 5 minutes, all the additions described previously were made to the supernatant fluid and the solution was then put into the Warburg vessel.   0.5 cc. of cysteine (final concentration 0.02 M) was added, since previous observations indicate that even under strictly anaerobic condition the yield is better in the presence of this amino acid (7).   The actual incubation time in all experiments was 20 minutes at 37°.   The amount of acetylcholine present was then tested on the frog rectus preparation.

Section of the sciatic nerve was carried out under ether anesthesia and under strictly sterile conditions.

Results

The experiments in which choline acetylase has been prepared from guinea pig and rat brain have shown that satisfactory results are obtained if the approximately 3 cc. of enzyme solution used per vessel contain the equivalent of 400 to 500 mg. of tissue.   The observations on cholinesterase indicate that the rate of acetylcholine metabolism is lower in the fibers than in the regions which contain cell bodies and synapses.   Consequently, it could be expected that the rate of acetylcholine metabolism would be lower in the fibers and that considerably less choline acetylase would be present there.   It therefore appeared necessary to use at least the same amount of tissue as in the experiments with brain extracts in order to obtain satisfactory data.

An advantage of the rabbit sciatic nerve as the material for testing the formation of acetylcholine by choline acetylase in normal and degenerated nerve fibers is the fact that its section is a simple operation.   From six to seven rabbits it is easy to obtain a total of 1.0 to 1.5 gm. of nerve (fresh weight) from both right and left sides.   This amount is sufficient to obtain solutions of suitable concentration for two vessels, one of which is used for the experiment in the presence of adenosine triphosphate, the second as a control without the nucleotide.

Only the difference between these two solutions (with and without

adenosine triphosphate) was used in the calculation of the acetylcholine formed. It is possible, however, that the small amounts of acetylcholine found in the control were also formed during incubation as a result of small amounts of preformed adenosine triphosphate. In that case the amounts actually formed would be slightly higher than those indicated.

Normal Nerve Fibers

The sciatic nerve contains a large amount of connective tissue. Since this tissue is rather tough and the grinding of the sciatic nerve and the preparation of a homogenized suspension are difficult, it takes some time and experience to achieve a complete or nearly complete extraction. In Table I the experiments are numbered in the chronological order in which they were carried out. It can be seen that in the later experiments higher yields were obtained than in the earlier ones. Whereas in the experiments up to No. 6 an average of 50 to 60 γ of acetylcholine is formed per gm. per hour, most of the later values obtained in Experiments 7 to 14 vary between 70 and 90 γ; that for Experiment 13 is 106. In the very first experiments in which choline acetylase was determined in the sciatic nerve, the values were even lower than those recorded in Table I. The yields of 70 to 90 γ per gm. per hour appear to be close to the highest that can be expected with the methods used.

The sciatic nerve contains a large amount of inactive tissue (connective tissue, fat, and myelin). On the assumption that this tissue forms about two-thirds of the total, which is a conservative estimate, the amount of acetylcholine which can be formed in the axon of the rabbit sciatic nerve may thus be about 250 γ per gm. per hour and is probably higher.

Degenerating Nerve Fibers—It appeared of special interest to determine the activity of choline acetylase during degeneration and to test how this metabolism is related to the nerve function; *i.e.*, to conductivity. Conduction is still maintained 2 days after section, whereas after 3 days it has disappeared. Obviously, if the release of acetylcholine is responsible for conductivity, formation of acetylcholine should be possible at a rate not too far below normal as long as the nerve is able to conduct. The disappearance of conductivity, on the other hand, may or may not be due to a loss of choline acetylase activity, since many essential enzymes or factors of an entirely different nature involved in and necessary for conductivity may be impaired before choline acetylase is inactivated.

48 hours after the section of the sciatic nerve, choline acetylase activity has decreased only about 20 to 25 per cent. It is obvious that Experiments 10 and 12 are more reliable than Experiments 3 and 5. In the former, the extraction was apparently complete, as the high normal value indicates. In the latter, the values are not yet maximal. Although it is probable that

in most cases the normal and the degenerated sides were ground in the same way, and that the relative values are therefore still comparable, as in Experiment 5, it is possible that the small decrease in Experiment 3 is due to slightly more efficient extraction of the degenerated side.

After 72 hours, the decrease is marked, but still about one-third of the enzyme is present. The slightly smaller decrease in Experiment 4 may be interpreted in the same way as was explained for Experiment 3.

6 days after section, choline acetylase activity is no longer detectable.

TABLE I

Choline Acetylase in Normal and Degenerating Rabbit Sciatic Nerves

The results of earlier experiments (Nos. 1 to 6), in which the extraction was probably not yet optimal, are put in parentheses.

Experi-ment No.	Time after section	Total weight		Equivalent weight used per vessel		Acetylcholine formed		Decrease
		Normal	Degen-erated	Normal	Degen-erated	Normal	Degen-erated	
	hrs.	gm.	gm.	mg.	mg	γ per gm. per hr.	γ per gm. per hr.	per cent
1	0	1.30	1.33*	322	328	(59.0)	(49.5)	
2	0	1.34	1.33*	395	392	(60.0)	(61.0)	
13	0	1.62	1.40*	461	401	106.0	106.0	
3	48	1.82	1.84	500	524	(53.0)	(51.0)	
5	48	1.54	1.84	530	586	(41.0)	(32.8)	
10	48	1.57	1.52	486	433	87.0	68.4	21.4
12	48	1.73	1.24	407	403	92.7	68.1	26.7
11	70	1.42	1.39	451	444	76.0	26.5	65.1
6	72	1.28	1.66	460	575	(52.0)	(17.0)	
4	72	0.94	1.41	340	495	(51.0)	(29.0)	
9	72	1.69	1.64	526	511	73.5	23.0	68.5
14	72	1.62	1.37	457	397	78.5	26.0	67.5
7	144	1.68	1.73	522	435	77.0	0	100
8	150	1.95	1.91	535	523	86.0	0	100

' Control.

DISCUSSION

Two facts emerge from these observations: First, the choline-acetylating enzyme system is present in a relatively high concentration in the peripheral axon which is free of cell bodies and nerve endings. This high rate of acetylcholine formation in the axon is consistent with the idea that the release of acetylcholine is responsible for the propagation of the nerve impulse along the axon as well as across the synapse. This finding is another important support for the assumption that no fundamental difference exists in the physicochemical mechanism of these two events, an

assumption for which a strong body of evidence already exists from biochemical as well as biophysical data.   It is difficult to believe that such a complex and specific enzyme system is present in the axon but has no function there, or that the function of the acetylcholine, formed at a high rate in the axon as well as at the synapse, changes suddenly when the impulse reaches the nerve ending.

The second essential fact is the evidence that the enzyme system has not markedly decreased after 48 hours, when the axon is still able to conduct impulses.   The decrease of 25 per cent is easily compatible with the assumption that this enzymatic mechanism is required for conduction. These results are in contrast to those of Feldberg (10) who recently claimed to have evidence for a synthesis of acetylcholine in the nerve fiber *in vitro* and that this synthesis becomes impossible 2 days after section of the fiber. He considers his results as an indication against the assumption of a rôle of acetylcholine in conduction.

The conditions and methods used by Feldberg are, however, inadequate for testing the synthesis of acetylcholine.   He worked under conditions in which there was no supply of energy necessary for the synthesis.   He kept chopped nerve fibers of cats and sheep for 2 hours in oxygenated Locke's solution, with the basic assumption that he had the same condition as did Quastel, working with brain slices.   There is, however, a decisive difference. Brain slices have a high rate of respiration, since the cells remain intact and there is, therefore, sufficient energy to build up adenosine triphosphate, whereas in chopped fibers the respiration disappears rapidly.   After 2 hours of incubation Feldberg found 1 to 2 γ of acetylcholine per gm. of nerve above the control.   We consider this difference to be due to the use of different methods of preparing the control and the experiment, which leads to slightly lower values in the control.   Indeed, Feldberg's figures show that if the two methods of extraction are compared without incubation the difference is the same as between control and incubated nerve.   His observations do not offer any evidence for a synthesis in the fiber and his failure to find any synthesis in degenerating nerve cannot be used as evidence against the concept presented above.

SUMMARY

Choline acetylase has been extracted from the rabbit sciatic nerve.   A solution prepared from 1 gm. of these nerve fibers forms 70 to 90 γ of acetylcholine per hour as compared with 150 to 200 γ per hour in solutions prepared from rat or guinea pig brains.

The presence of the complex energy-requiring enzyme system in the nerve axon, *i.e.* that part of the neuron which does not contain nerve endings and cell bodies, is further support for the assumption that acetyl-

choline may not only be essential for the transmission of the nerve impulse across the synapse but also for its propagation along the axon.

In the degenerating nerve fiber the enzyme activity decreases slowly. 48 hours after section only 20 to 25 per cent of the initial activity is lost. At that time conductivity is still possible. 72 hours after section, when conductivity has disappeared, the enzyme activity has decreased by two-thirds. No enzyme was found 6 days following section. The results are consistent with the assumption that the release of acetylcholine is associated with conductivity.

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REVERSAL OF THE ACTION OF PHENYL PANTOTHENONE BY CERTAIN AMINO ACIDS

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Phenyl pantothenone, the phenyl ketone analogue of pantothenic acid, has been shown to cause inhibition of microbial growth, and this action has been found to be reversible competitively by pantothenic acid (1). However, the antagonism exerted by pantothenic acid occurred only in the case of organisms which were stimulated to grow by that vitamin; those species which synthesized their own supply of the growth factor were not protected against the toxic action of phenyl pantothenone by the vitamin. A study has now revealed that there is in natural products something which will antagonize the action of phenyl pantothenone in these latter species.

Attempts were made to isolate and identify this active constituent of natural products, for it was hoped that a knowledge of its nature might furnish some clue to the mode of action of pantothenic acid. A survey showed that one of the best sources of the factor was an acid hydrolysate of casein. Fractionation of this material indicated that potency resided in the butanol residue after repeated extraction of an aqueous solution, and, since arginine, histidine, lysine, glutamic acid, aspartic acid, serine, threonine, and glycine are known to occur in such a fraction, these amino acids were tested for their ability to reverse the antimicrobial action of phenyl pantothenone.

Some of these amino acids were active in this regard. Histidine was the most effective, but, since it was rather toxic for the test organism, there was only a narrow range between effective and toxic doses. Glutamic acid was quite active and proline, glycine, aspartic acid, or asparagine was less so. Alanine, lysine, serine, or threonine showed some activity, but the remaining known amino acids were unable to protect against the inhibition caused by phenyl pantothenone. Those amino acids which were effective were each about as potent singly as in combination with each other.

EXPERIMENTAL

Method of Assay—The synthetic basal medium used previously (1) for the growth of *Saccharomyces cerevisiae* was supplemented with 0.14 γ of calcium pantothenate and 160 γ of phenyl pantothenone per cc. In this medium little if any growth of *Saccharomyces cerevisiae* occurred under the condi-

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* With the technical assistance of Ruth A. Brown.

tions of assay, but, if the phenyl pantothenone was omitted, very good multiplication resulted (see Table I). As reported earlier (1), additional amounts of pantothenic acid were unable to nullify the action of phenyl pantothenone in this basal medium. The inoculum and conditions of incubation and the manner of estimating growth were the same as used before. Time of incubation was 20 to 24 hours. Materials to be assayed were added in graded doses to this basal medium, and, if growth occurred when this was done, it was concluded that an antagonist to phenyl pantothenone was present.

Natural Occurrence of Antagonists to Phenyl Pantothenone As Measured with Saccharomyces cerevisiae —Bacteriological broth or peptone caused complete reversal of phenyl pantothenone inhibition of growth at about 5 mg. per cc. of culture. Liver extract had approximately the same po-

TABLE I

Effect of Glutamic Acid on Growth of Saccharomyces cerevisiae in Presence of Phenyl Pantothenone and Pantothenic Acid

d Glutamic acid	Phenyl pantothenone	Turbidity*
mg per cc	γ per cc	
0	0	42
0	160	98
0 4	160	98
0 8	160	94
1 6	160	64
3 3	160	41
1 6	0	29
0 4	0	31

* Expressed as per cent of the incident light transmitted.

tency, while "tryptone" was about twice as effective. An acid hydrolysate of vitamin-free casein gave half maximal reversal at 1.2 mg. per cc., and complete counteraction at 2 mg.

Fractionation of Casein Hydrolysate – When acid-hydrolyzed casein (S. M. A. Corporation) in 5 per cent solution at pH 6 was extracted ten times with butanol, the extracted material was found to be inactive when tested at a level equivalent to an effective dose of the starting material, while the butanol residue contained all the activity. Phosphotungstic acid divided the activity between precipitate and filtrate. Ionic exchange resins (Amberlite), designed for the removal of bases, adsorbed only part of the active material from solution. These facts suggested that a mixture of amino acids might be involved, and that the butanol-extractable acids were not primarily responsible. These suppositions were shown to be correct by tests with pure compounds.

Reversal of Action of Phenyl Pantothenone by Various Amino Acids—In Table II are listed the amounts of the various amino acids which caused half maximal stimulation of growth (*i.e.*, half maximal reversal) in the presence of phenyl pantothenone. Histidine was the most active, but levels above 1.6 mg. per cc. of culture were completely inhibitory of growth of *Saccharomyces cerevisiae* even in the absence of phenyl pantothenone. In view of this fact, glutamic acid was the most suitable reversing agent. The *dl* form possessed just half of the activity of the optically active acid. This also was true of proline.

The antagonism between phenyl pantothenone and glutamic acid could

TABLE II

Effect of Various Amino Acids on Growth of Saccharomyces cerevisiae Inhibited by Phenyl Pantothenone

The tests were performed in the presence of 160 γ of phenyl pantothenone and 0.14 γ of pantothenic acid per cc.

Amino acid	Amount required for half maximal growth	Amino acid	Amount required for half maximal growth
	mg. per cc.		*mg. per cc.*
1. Glycine	2.6	12. *l*-Aspartic acid	2.5
2. *dl*-Alanine	>3.0*	13. *l*-Asparagine	2.5
3. *dl*-Valine	No effect at 3.4	14. *d*-Glutamic acid	1.2
4. *dl*-Isoleucine	" " " 3.4	15. *dl*-Glutamic acid	2.4
5. *dl*-Leucine	" " " 3.4	16. *l*-Proline	2.3
6. *dl*-Phenylalanine	" " " 3.4	17. *dl*-Methionine	No effect at 3.4
7. *dl*-Serine	>3.0	18. *l*-Cystine	" " " 3.4
8. *dl*-Threonine	>3.0	19. *dl*-Tryptophane	" " " 3.4
9. *d*-Arginine	No effect at 3.4	20. *l*-Tyrosine	" " " 3.4
10. *d*-Lysine	>3.4	(7 + 8 + 12 + 14)	1.3
11. *l*-Histidine	0.26	(1 + 2 + 16)	2.4

* This denotes some activity but indicates that half maximal growth was not produced by the greatest concentration of amino acid tried.

be demonstrated only over a limited range of concentration. For example, half maximal effect was noted with 0.12 mg. of the glutamic acid in the presence of 80 γ of the vitamin analogue, and with 1.2 mg. of the acid in the presence of 160 γ of the analogue. A point was soon reached (as the concentration of phenyl pantothenone was raised) beyond which it was not possible to cause reversal. For example, with 600 γ of phenyl pantothenone no detectable effect was produced by 5 mg. of glutamic acid.

The results of the tests were complicated because of the fact (2) that many of the amino acids caused slight acceleration of growth of *Saccharomyces cerevisiae* in the basal medium in the absence of phenyl pantothenone. However, the concentrations needed for maximal growth-promoting effect

were much less than those involved in the reversal of the action of phenyl pantothenone (see Table I).   It was therefore possible to exclude this cause of error by additions of small amounts of a given amino acid to the test medium.

Antagonism between Amino Acids and Phenyl Pantothenone in Lactobacillus casei—Since the antagonism between amino acids and phenyl pantothenone had been established with an organism which did not require preformed pantothenic acid in the growth medium, experiments were done to determine whether similar relationships held for species which needed this vitamin, and in which competition between phenyl pantothenone and pantothenic acid could be demonstrated.   Trials with *L. casei*, one of this second class of species, showed that the situation was much like that with *Saccharomyces cerevisiae* in that glutamic acid and glycine reduced the toxicity of phenyl pantothenone, and that the former amino acid

TABLE III

Effect of Glutamic Acid on Inhibition of Growth of Lactobacillus casei Caused by Phenyl Pantothenone

d-Glutamic acid	Phenyl pantothenone required to cause half maximal inhibition
mg. per cc.	*γ per cc.*
1.2*	18
3.2	22
5.2	34
9.2	36

* This was the amount of glutamic acid contained in the basal medium.

was more active in this respect than was the latter.   The tests were conducted by determining the amount of the inhibitor (phenyl pantothenone) which would cause half maximal inhibition of growth in the presence of varying amounts of the amino acid.   The medium and procedure involved have been described (1).   Glutamic acid was present in the basal medium, since *L. casei* failed to grow without it (3), but additional quantities of this amino acid decreased the toxicity of phenyl pantothenone (see Table III). Complete reversal of the effect of a fully growth-suppressing dose of the inhibitor could not be achieved by glutamic acid.   This was in contrast to the results with *Saccharomyces cerevisiae*.

DISCUSSION

The antagonism between the vitamin analogue, phenyl pantothenone,

and the structurally dissimilar amino acids such as histidine, glutamic acid, and proline illustrates a phenomenon which is not unique in the relationships of metabolites and their competing analogues. For example, the action of the sulfonamides is reversed by the analogous metabolite p-aminobenzoic acid, but also by the structurally dissimilar amino acid methionine (4), or by adenine (5). Furthermore, the biological effects of 3-acetylpyridine are negated by the related metabolite nicotinic acid, but just as well by the unrelated amino acid tryptophane (6, 7). In like manner, the manifestations produced by glucoascorbic acid are prevented by ascorbic acid in guinea pigs (8), and by a substance in certain plants (9). This material appears to be of protein or peptide nature, since it is destroyed by proteolytic enzymes (unpublished data). The situation with glucoascorbic acid is similar to the one with phenyl pantothenone, for in both cases the inhibitor was reversed in its action by the related vitamin only in those species which were unable to synthesize their own supply of the vitamin; for those that could perform this synthesis, the effects of the inhibitor could be antagonized only by the structurally dissimilar substance.

From these examples it appears that, in general, inhibitory structural ·analogues, which behave competitively with their related vitamin or other metabolite, may be antagonized in their action by structurally dissimilar natural substances as well as by the structurally related metabolite. Frequently these other antagonists are amino acids.

SUMMARY

The antimicrobial action of phenyl pantothenone, a structural analogue of pantothenic acid, was reversed by certain amino acids. This antagonism with amino acids was found with all species examined, while, as was known previously, the antagonism with pantothenic acid was operative only in those organisms which were stimulated in growth by the vitamin. For *Saccharomyces cerevisiae*, histidine was the most active amino acid in causing reversal of the inhibition of growth produced by phenyl pantothenone. Glutamic acid was next in line of activity, while proline, aspartic acid, asparagine, and glycine were less effective. Some potency was displayed by serine, threonine, alanine, and lysine but other amino acids were not effective. Similarities of the relationship of phenyl pantothenone, pantothenic acid, and certain amino acids to that of the sulfonamides, p-aminobenzoic acid, and methionine, and of that of 3-acetylpyridine, nicotinic acid, and tryptophane have been pointed out.

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THE RIBONUCLEINASE OF THE SOY BEAN

I. ISOLATION OF THE ENZYME*

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The hydrolysis of nucleic acids by direct chemical methods is recognized as a rather unsatisfactory procedure for the preparation of nucleotides and nucleosides in that the mixtures obtained are difficult to separate and the yields of the desired products are low. Enzymatic hydrolysis should prove applicable to this problem and has been used, with limited success, for obtaining degradation products of both ribonucleic and desoxyribonucleic acids (1–4). Owing to the difficulties attending the preparation of the necessary enzymes, however, enzymatic methods of hydrolysis have not been widely used.

· The enzymes which effect the hydrolysis of the nucleic acids and their intermediate breakdown products have been grouped together under the name *nucleases* and, according to Levene and Medigreceanu (5) and Bredereck (6), fall into three classifications according to the substrates upon which they act. These are, respectively, the *polynucleotidases*, the *nucleotidases*, and the *nucleosidases*. No attempt will be made here to review the literature concerning the nucleases. It should be pointed out, however, that there is considerable confusion in this literature concerning the specificity and the nomenclature of these enzymes. Thus, the active agent which effects the rupture of the ribonucleic acid molecule without the release of inorganic phosphate has been referred to by various workers as *ribonucleodepolymerase* (7), *ribonuclease* (8), *polynucleotidase* (9), *ribonucleinase* (10), and even as *nucleotidase* (11).[1] Some of this confusion arises naturally from the fact that the enzyme preparations were not pure and exhibited different degrees of specificity. There has been no recent and systematic investigation of the nucleases from the point of view of

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* A part of a thesis submitted by Max Schlamowitz to the Graduate School of the University of Michigan in partial fulfilment of the requirements for the degree of Doctor of Philosophy.

[1] In this paper we have used the term *nuclease activity* as indicative of the presence of enzymes which promote the hydrolysis of ribonucleic acid with the formation of inorganic phosphate. The term *ribonucleinase* is used to designate an enzyme which brings about the formation of degradation products of ribonucleic acid in which the phosphorus is retained in organic combination. For a justification of this name see Loring and Carpenter (10).

modern enzyme and protein chemistry and, with the exception of the ribonucleinase of the pancreas which has been obtained by Kunitz (8) in a crystalline form, no member of the nucleases has been prepared in a well defined state of purity.

This paper presents the results of the first of a series of studies in which it is planned to isolate and, if possible, to purify the components of a system of nucleases. The purpose of the research is twofold: to study the chemical composition and specificity of the enzymes, and to attempt their preparation in a quantity sufficient to serve for fairly large scale hydrolyses of ribonucleic acid.

The starting point of the investigation was the selection of a source of the enzymes. In 1930, Jono (12) reported that the seeds, stems, and leaves of a number of plants contained nucleases. Among the species investigated, the common soy bean exhibited a comparatively high activity in the hydrolysis of ribonucleic acid. The nuclease activity was evidenced by the increase of inorganic phosphate in the mixtures of the ribonucleic acid and extracts of the beans. Since the soy bean is readily available in quantity, it was decided to reinvestigate this reported nuclease activity. In so far as it has been possible to determine, there has been no mention in the literature of the soy bean nuclease apart from this initial report.

Our first experiments confirmed the finding that extracts of germinated soy beans were capable of promoting the hydrolysis of ribonucleic acid. A number of attempts were made to purify and to concentrate this nuclease activity but these were uniformly unsuccessful in that they led to preparations which exhibited no greater activity than did the original crude extracts. Frequently the activity was entirely destroyed by relatively mild treatment. The techniques employed in these experiments included precipitations with organic solvents, salting-out procedures, and attempts to adsorb and elute the activity with various inert adsorbents. These experiments will not be reported in detail here, as they led to little information concerning the nuclease activity.

In order to explain these failures to concentrate the nuclease activity, it was assumed that the observed breakdown of the ribonucleic acid might be due to the combined actions of more than one enzyme. Subsequent studies have proved the presence of two enzymes in the crude extracts. The first of these has been identified as a *ribonucleinase* whose action is to convert ribonucleic acid into products which are soluble in glacial acetic acid but which retain the phosphorus of the substrate in organic combination (10). The second enzyme is responsible for the formation of the inorganic phosphate in the substrate-extract mixtures and is believed to be a *phosphomonoesterase*. It has not been well studied, as it is destroyed in the course of the isolation of the nucleinase.

Ribonucleinase has been isolated in a crystalline form from pancreatic tissue (8) and has been detected in blood (13), bone marrow (14), spleen (14), and *Pasteurella pestis* (15). We have now demonstrated its occurrence in the higher plants by the preparation of highly active solutions from soy bean seedlings. From this pattern of distribution it might be concluded that ribonucleinase is an important agent in intercellular metabolism. The products of its action upon ribonucleic acid are, however, not definitely known (15) and its physiological function within the cell must be clarified by further research.

The work reported here is primarily concerned with the proof of the occurrence and the isolation of the nucleinase. The preparation of the enzyme in larger quantities and the more detailed investigation of its reaction characteristics are in progress in this laboratory and will be the subject of a later communication.

EXPERIMENTAL

Materials– The nucleic acid used in these studies was a commercial preparation of yeast sodium nucleate.[2] In the earlier experiments this material was used without purification. Later it was found that the preparation contained material, presumably of the nature of mononucleotides, which inhibited nucleinase activity. For all subsequent work, therefore, the yeast sodium nucleate was converted into ribonucleic acid and purified according to the procedure of Kunitz (8). After this work was completed and was being prepared for publication a report by Zittle (16) appeared which emphasizes the presence of nucleinase-inhibiting substances in some of the commercially available nucleic acids.

The soy beans were a common variety of field beans, *Glycine hispida* var. Manchu.[2] The beans were sprouted under conditions which discouraged mold growth and the seedlings were macerated and extracted with a 40 per cent solution of glycerol. The following procedure has been found satisfactory for the sprouting of the beans and the preparation of the extracts.

A weighed quantity of the soy beans was washed with a 0.05 per cent solution of calcium hypochlorite. They were thoroughly rinsed with distilled water and permitted to stand in water overnight. The swollen beans were again washed with the calcium hypochlorite solution and thoroughly rinsed with distilled water. They were then placed in wide mouth reagent jars, each of which received approximately 150 gm. of the swollen beans. The jars were closed with a double layer of cheese-cloth

and were supported in an inverted position to facilitate drainage of excess water. The germination was carried out in a dark room at a temperature between 20–25°. At approximately 8 hour intervals, the jars were filled with water and emptied, a procedure which served to maintain a moist atmosphere and to wash out developing bacteria. After 96 hours the sprouts were between 3 and 4 cm. in length. Under the conditions described a germination of nearly 100 per cent was obtained and no mold growths or other visible contaminations were encountered.

The sprouted beans were removed from the jars and weighed. They were then mixed with glycerol and water in proportions which were calculated in the following manner. Each 100 gm. of the dry beans were to be extracted with 750 ml. of 40 per cent glycerol. Since a considerable portion of the required water had been absorbed by the beans in the course of the germination process, the difference between the weights of the dry and germinated beans was subtracted from the calculated water requirement. The exact procedure is illustrated by a typical example.

Dry beans, 500 gm., were calculated to require 3750 ml. of extraction medium to be composed of 1500 ml. of glycerol and 2250 ml. of water. The weight of the sprouted beans was 1550 gm., of which 1050 gm. were water absorbed in the process of germination. The sprouted beans were, therefore, mixed with 1500 ml. of glycerol and 1200 ml. of water. This mass was thoroughly homogenized in a Waring blendor and placed in the refrigerator for 48 hours. The fluid was then pressed out through cheesecloth and the extract was centrifuged. The heavy, cream-colored supernatant fluid was covered with toluene and stored in the refrigerator. This material is referred to as the *crude extract* from which all subsequent enzyme preparations were derived. When preserved as indicated, it retains its activity for many months.

Methods—The nuclease activity of the various preparations was measured by the determination of the increase of inorganic phosphate in the mixtures of the extract and ribonucleic acid. Since some of the degradation products of the ribonucleic acid react with molybdic acid, it was necessary to carry out a preliminary precipitation of the phosphate before it was determined by the Fiske-Subbarow method (17).

A 5 ml. aliquot of the enzyme-substrate mixture was pipetted into 5 ml. of a 20 per cent solution of trichloroacetic acid contained in a conical centrifuge tube. The mixture was stirred for several minutes and was centrifuged. An appropriate aliquot of the supernatant fluid, usually 5 ml., was mixed with 5 ml. of magnesia mixture (18), and the acidity of the solution was adjusted to pH 5.5 by the careful addition of concentrated ammonium hydroxide. 1 ml. of the ammonium hydroxide in excess was added and the mixture was placed in the refrigerator for 24 hours. The ammonium

magnesium phosphate was centrifuged and was washed once with 1.5 ml. of ice-cold 2 per cent ammonium hydroxide. The washed precipitate was dissolved in 1 ml. of the acid solution, Molybdate II, of Fiske and Subbarow (17) and the solution was quantitatively transferred to a 25 ml. volumetric flask. The aminonaphtholsulfonic acid solution (17) was added and the solution was made up to volume. After 5 minutes the intensity of the color was determined with a photoelectric colorimeter.

The determination of ribonucleinase activity was based upon the fact that the intact nucleic acid molecule is precipitated by glacial acetic acid, whereas the products of nucleinase action are soluble in this reagent (8, 15). Aliquots of the material to be analyzed were pipetted into 5 times their volume of glacial acetic acid. The mixtures were filtered and the total phosphorus in the filtrates was determined by the perchloric acid method of King (19). The increase in the total phosphorus soluble in glacial acetic acid, appropriately corrected for the controls, was a convenient measure of the ribonucleinase activity.

Expression of Enzyme Activity—As no detailed study has been made of the phosphomonoesterase, the nuclease activity of the crude extracts has been expressed simply as the increase in phosphate phosphorus per ml. of the incubation mixture.

The nucleinase unit employed in the later experiments is defined as that amount of the enzyme which will bring about an increase of 1 mg. of acid-soluble phosphorus in 24 hours in a mixture of the enzyme and a 1.0 per cent solution of ribonucleic acid at pH 6.2 at 30°. The total nitrogen of each enzyme solution was determined by the micro-Kjeldahl method (20) and the amount of an enzyme preparation used in a particular experiment is expressed in terms of its content of nitrogen. Willstätter and Kuhn (21) have emphasized that the expression of the concentration of an enzyme should be in terms of the number of units contained in a given amount of the preparation, a quantity which they term the *enzyme value*. We have calculated our results in terms of the *ribonucleinase value* which is defined as the number of ribonucleinase units per mg. of nitrogen present in the enzyme preparation. The ribonucleinase value affords a convenient basis for the comparison of the activity and degree of purity of the various enzyme preparations.

Results

Nuclease Activity of Soy Bean Extracts— The selection of germinated soy beans as the better source of the nuclease activity was made upon the basis of a comparison of the activities of extracts of dormant and sprouted beans.

Two enzyme solutions were prepared. The first was a 40 per cent glycerol extract of 5 gm. of finely ground soy beans. The second was a

40 per cent glycerol extract of 5 gm. of beans which had been sprouted in
the manner described above. The volumes of the two extracts were ap-
proximately equal. Duplicate experiments were set up in which 2 ml. of
the appropriate extract were mixed with 40 ml. of a 1.0 per cent solution
of yeast sodium nucleate in 0.02 M citrate buffer of pH 6.2. Control tubes,
two of which contained only the enzyme solutions and the third of which
contained the buffered substrate alone, were incubated simultaneously with
the experimental mixtures at 30°. At approximately 24 hour intervals ali-
quots of the various solutions were withdrawn and analyzed for their content
of inorganic phosphate in the manner which has been described. The results
of the analyses were calculated in terms of increases in inorganic phos-
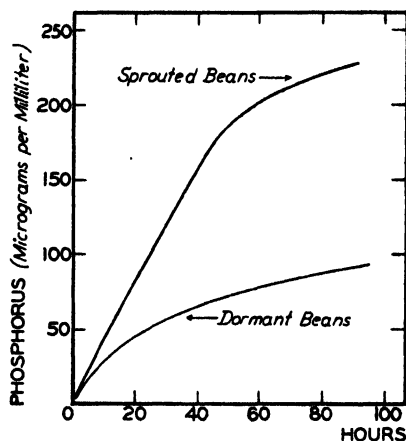
FIG. 1. Comparison of the nuclease activities of dormant and sprouted soy beans

phate per ml. of solution, appropriately corrected for the changes observed
in the control tubes.

The results of the experiment are presented in Fig. 1. It is evident
that the crude extract of the sprouted beans contained a considerably
greater nuclease activity than did the extract of the dry beans. It is not
implied that the enzymes present in the two extracts are identical; the
experiment merely indicated that germinated beans contained a greater
concentration of nuclease activity and for this reason subsequent work
was carried out upon the sprouted beans.

Demonstration of Dual Nature of Nuclease System—It was mentioned
in an earlier paragraph that the usual methods for the concentration of
enzyme solutions were not applicable to the purification of the nuclease
activity of the crude soy bean extracts. In the course of these attempts to
purify the extracts, however, it was observed that the trichloroacetic acid
filtrates prepared from the control tubes which contained only ribonucleic

acid were usually turbid, whereas the corresponding filtrates from the experimental mixtures were frequently clear. Although the turbidity interfered in no way with the determination of the inorganic phosphate, consideration of the phenomena led to the conclusion that the turbidity was due to the precipitation of unaltered ribonucleic acid in a very finely divided form. The fact that the filtrates from mixtures which contained the enzyme preparations were frequently clear led to the speculation that the ribonucleic acid had been altered by the extracts, although nuclease activity was not evident from the results of the analyses for inorganic phosphate.
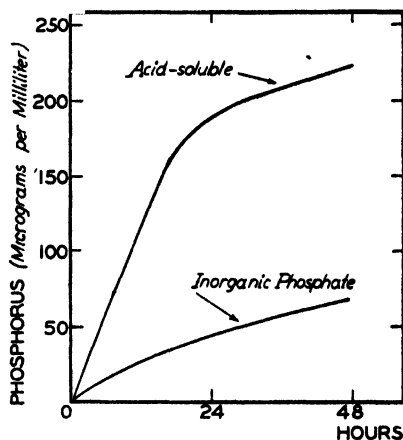
FIG. 2. Comparison of the nuclease and ribonucleinase activities of soy bean extracts.

Accordingly, an experiment was set up in which each aliquot of the ribonucleic acid-soy bean extract mixture was analyzed for acid-soluble phosphorus as well as for its content of inorganic phosphate. The details of the experiment were identical with those described above for the detection of the nuclease activity of the extract of the germinated seeds. The results are presented in Fig. 2 from which it is evident that the extract was capable of forming acid-soluble phosphorus from the ribonucleic acid at a greater rate than it released inorganic phosphate. The experiment was considered as indicative of the presence of two enzymes in the crude extract, one of which was responsible for the increase in the acid-soluble phosphorus, and the second for the formation of the inorganic phosphate.

Isolation of Ribonucleinase—A number of orientation experiments were carried out in an attempt to determine the best method for the separation of the two enzymes. It was found that the phosphomonoesterase activity was sensitive to acid, whereas the ribonucleinase activity was not only

stable to acid but was also moderately resistant to inactivation by heat.[3] The following procedure has served to prepare ribonucleinase solutions of a good degree of activity.

The crude glycerol extract of the sprouted soy beans, 1500 ml., was diluted with an equal volume of water and the acidity adjusted to pH 4.0. The solution was placed in the refrigerator for 3 hours and was then partially neutralized by the addition of concentrated ammonium hydroxide to pH 4.8. The preparation was placed in the refrigerator for 12 hours and was then centrifuged. The precipitate was discarded. The solution was placed in cellophane bags and dialyzed against cold, running distilled water for 48 hours. The preparation was removed from the

TABLE I

Activity of Concentrated Ribonucleinase

1 ml. of the concentrated enzyme was added to 20 ml. of each substrate. Commercial substrate refers to a 1.0 per cent solution of the yeast sodium nucleate in 0 02 M citrate buffer of pH 6.2 and the purified substrate refers to a 1.0 per cent solution of reprecipitated ribonucleic acid which had been neutralized with sodium hydroxide and made up in the citrate buffer. The experimental and control tubes were incubated at 30°. The values listed are in terms of micrograms of phosphorus per ml., appropriately corrected for the controls

Incubation time	Substrate	Nuclease activity (inorganic phosphate)	Ribonucleinase activity (acid-soluble phosphorus)
hrs.			
0	Commercial	0.0	0.0
0	Purified	0.0	0.0
24	Commercial	34.2	270
24	Purified	9.9	470
48	Commercial	52.5	292

dialysis sacs and was filtered from the slight sediment which had formed in the course of the dialysis. The filtrate was concentrated *in vacuo* to a volume of 380 ml. During the concentration the temperature was not permitted to go above 30° and the pressure varied from 12 to 17 mm. of mercury. The resulting solution has been designated the *concentrated ribonucleinase solution.*

Solutions prepared in this manner exhibited strong ribonucleinase activities and contained most of the enzyme present in the original crude extracts. The removal of the phosphomonoesterase activity was more or less complete, as evidenced by the low values which were obtained in the assays for nuclease activity.

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[3] The ribonucleinase of the pancreas has been shown to be remarkably stable to heat inactivation (8, 22).

In the course of the determination of the activity of these concentrated ribonucleinase solutions, it was observed that the commercial yeast sodium nucleate contained material which inhibited the action of the nucleinase. An illustration of this inhibition is seen in Table I in which the assay of one preparation of the ribonucleinase was made against the commercial substrate as well as against a sample of purified ribonucleic acid. These data not only show the inhibition of the ribonucleinase but also indicate that this material, presumably of mononucleotide nature (16), leads to an enhanced value for the monophosphatase activity. This latter effect is possibly due to the fact that the presence of mononucleotides in the substrate furnished material upon which the monophosphatase acts readily. In all subsequent work the substrate of choice was the purified ribonucleic acid.

Fractionation of Concentrated Ribonucleinase by Precipitation with Ammonium Sulfate

Concentrated nucleinase
  | Make 0.5 saturated with $(NH_4)_2SO_4$; filter

Filtrate
  | Dialyze 36 hrs.

Fraction B
  | Make 0.8 saturated with $(NH_4)_2SO_4$; filter; discard filtrate; dissolve ppt. in water; make 0.45 saturated with $(NH_4)_2SO_4$; filter

Ppt.
  | Dialyze 36 hrs.; dissolve in water

Fraction A
  | Make 0.5 saturated with $(NH_4)_2SO_4$; filter

Ppt.
  Dissolve in water; dialyze 36 hrs.; filter; discard ppt. Solution is Fraction X

Filtrate
  Increase $(NH_4)_2SO_4$ concentration to 0.8 saturation; discard filtrate; dissolve ppt. in water; dialyze 48 hrs.; filter; discard ppt. Solution is Fraction Y

Filtrate
  | Dialyze 48 hrs.

Fraction C
  | Make 0.6 saturated with $(NH_4)_2SO_4$; filter

Ppt.
  Dissolve in water; dialyze 48 hrs ; filter; discard ppt. Solution is Fraction D

Filtrate
  Increase $(NH_4)_2SO_4$ concentration to 0.8 saturation; filter; discard filtrate; dissolve ppt. in water; dialyze 48 hrs.; discard ppt. Solution is Fraction F

Ppt.
  Dissolve in water; dialyze 48 hrs.; discard ppt. Filtrate is Fraction E

Further Purification of Ribonucleinase—The concentrated ribonucleinase solutions were further purified by fractional precipitation with ammonium sulfate. The accompanying scheme shows this fractionation which consisted of the addition of ammonium sulfate to the desired concentration, removal of the precipitated protein, dialysis of the filtrate and of the redissolved precipitate, and refractionation of these two solutions. All of these operations were carried out between 5–10°. The various fractions thus obtained were assayed for ribonucleinase activity in the usual manner.

TABLE II

Distribution of Ribonucleinase Activity between Fractions Obtained by Precipitation with Ammonium Sulfate

The data summarize two experiments, Nos. I and II. The fractions refer to the preparations described in the scheme. The substrate was 20 ml. of 1.0 per cent solution of purified ribonucleic acid in 0.02 M citrate buffer of pH 6.2. The enzyme was added to the substrate in quantities of 0.2 to 1.0 ml , depending upon the activity of the fraction. Enzyme concentrations are given in terms of micrograms of enzyme nitrogen per ml. of the incubation mixture. The ribonucleinase activity figures are in terms of micrograms of increased acid-soluble phosphorus per ml. of incubation mixture, appropriately corrected for the controls. The incubations were carried out at 30°. The significance of the ribonucleinase value is discussed in the text

Enzyme preparation	Enzyme concentration		Ribonucleinase activity		Ribonucleinase value	
	Experiment I	Experiment II	Experiment I	Experiment II	Experiment I	Experiment II
Concentrated enzyme	43		470		10.9	
Fraction A	34 5		231		6.7	
" B	8.7		296		34.2	
" X	26.4	52.7	139	179	5.3	3 4
" Y	59.5	20.2	461	104	7 7	5.1
" C		2.9		315		108 6
" D	15 4	59.9	453	434	29 4	7.2
" E	5.6	3.3	460	171	82.1	51.8
" F	4.4	1.56	604	373	137.3	239.1

The results of typical fractionations of two preparations of the concentrated nucleinase are shown in Table II. It is evident that the separation of the enzyme from inert material is not particularly sharp and that the ribonucleinase activity is to be found in several of the fractions. The most active material is precipitated between the concentrations of 0.5 and 0.8 saturation with ammonium sulfate and has been designated Fraction F. One preparation of Fraction F had a nucleinase value of 239. The corresponding value for the crude extract from which this preparation was derived was not determined but calculations based upon the dry

weights of these crude extracts indicate that their nucleinase values are less than 1.0. It is reasonable to assume that a concentration ratio of at least 240 has been effected. None of the fractions obtained by the ammonium sulfate precipitation exhibited a release of inorganic phosphate from the substrate.

SUMMARY

The nuclease system of the soy bean seedling has been shown to consist of two components, a ribonucleinase and a second enzyme, believed to be a phosphomonoesterase. The ribonucleinase has been separated from the crude extracts and has been concentrated and partially purified.

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AN IMPROVED MEDIUM FOR MICROBIOLOGICAL ASSAYS WITH LACTOBACILLUS CASEI

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Since its introduction as an assay organism for riboflavin (1), *Lactobacillus casei* has been widely used for the determination of this and other vitamins which it requires (2–6). Landy and Dicken (3) devised a single medium with which responses to each of the six vitamins required by this organism could be obtained. Experimental trial showed the medium to be lacking in substances which, though not essential, greatly stimulated growth of *Lactobacillus casei*. Because other special methods for which this was not so obviously true existed, the more general method of Landy and Dicken has not been widely used.

. Recent investigations have shown that tryptic digests of casein (7–9) and of other proteins (10) contain one or more unidentified substances which greatly stimulate growth of *Lactobacillus casei*. This knowledge has been applied to the development of an improved medium for this organism, which is described below. Maximum growth on the improved medium is achieved within 16 hours; longer periods are required for maximum acid production. Excellent growth curves have been obtained in response to the addition of any one of five vitamins to the appropriate deficient medium. Results of a series of assays for riboflavin and folic acid indicate that the medium can be used successfully for the accurate estimation of these vitamins after a 16 hour incubation period. Similar determinations of biotin, pantothenic acid, and nicotinic acid appear possible, although such application has not been tested.

EXPERIMENTAL

Enzymatic Casein Digest—120 gm. of purified casein (Labco, "vitamin-free") were dissolved by gradual addition, with shaking, to 2 liters of 0.8 per cent sodium bicarbonate solution. To the uniform suspension thus obtained were added 600 mg. of pancreatin suspended in 15 to 20 cc. of distilled water. The mixture was covered with a thin layer of toluene, shaken well, and allowed to stand for 48 hours at 37°. It was then placed in the autoclave and heated in flowing steam for 20 to 30 minutes to remove the toluene. After cooling to room temperature, the solution was adjusted to pH 6.0 by addition of glacial acetic acid (about 7.0 cc.), and filtered with

mild suction. To the filtrate were added 60 gm. of activated charcoal (Darco G-60); the mixture was stirred for 30 minutes, then filtered with suction. The filtrate, which often contained small amounts of colloidal charcoal, was now adjusted to pH 3.8 with glacial acetic acid (about 75 cc.), stirred for 30 minutes with 24 gm. of activated charcoal, and filtered. The filtrations are aided by the use of Filter-Cel. The residue from each filtration was washed with 50 to 75 cc. of water; the washings were added to the filtrate.

TABLE I

*Composition of Complete Basal Medium**

Component	Amount per 10 cc final medium	Component	Amount per 10 cc. final medium
	cc		γ
Casein digest	4 ($\approx$ 200 mg. casein)	p-Aminobenzoic acid	1
	mg		
Sodium acetate†	200	Biotin	0 04
KH₂PO₄	25	Calcium pantothenate	5
K₂HPO₄	25	Folic acid	0 02
Glucose	200	Nicotinic acid	5
Cystine	1	Pyridoxine hydrochloride‡	10
	γ		
Uracil	100	Riboflavin	5
Adenine sulfate	100	Thiamine hydrochloride	5
			cc.
Guanine hydrochloride	100	Salts C§	0 2

* For use in assays, the appropriate vitamin is omitted from the medium

† When the casein digest is prepared as described in the text, it contains the proper amount of sodium acetate, no further additions should be made

‡ Pyridoxamine (2 γ) or pyridoxal (1 γ) may be substituted for pyridoxine

§ The solution of Salts C has the following composition: $MgSO_4 \cdot 7H_2O$, 10 gm ; NaCl, 0.5 gm ; $FeSO_4 \cdot 7H_2O$, 0.5 gm.; $MnSO_4 \cdot 4H_2O$, 2.0 gm.; water to make 250 cc. It differs quantitatively from Salts B used in previous assay media

These charcoal treatments serve to remove traces of the various vitamins which are present in the initial digest. The final clear filtrate was diluted to 2.4 liters (1 cc. $\approx$ 50 mg. of casein). Dry weight determinations on small aliquots of each lot of casein digest prepared in this way showed 40 to 45 mg. of solids actually present per cc. of solution. If markedly lower amounts are obtained, the preparation should be repeated.

Basal Medium--The composition of the basal medium is shown in Table I. The vitamin to be determined is omitted from the medium. The

method used to obtain the final medium is immaterial. An efficient means for accomplishing this consists in preparing and keeping separately three stock solutions which are mixed in the proper proportions just before use. These are as follows: (a) A solution containing the casein digest, sodium acetate, phosphates, cystine, and Salts C. This is prepared in lots of 2 liters at twice the final concentration of the medium by adding to 1600 cc. of the casein digest solutions containing 10 gm. of K_2HPO_4, 10 gm. of KH_2PO_4, 400 mg. of cystine, and 80 cc. of Salts C. The mixture is neutralized (pH 6.8 to 7.0) with 10 N NaOH and diluted to 2 liters. The sodium acetate thus formed brings its concentration to the proper level if acetic acid was used in the amounts indicated in preparing the casein digest. The solution was autoclaved for 10 minutes at 15 pounds steam pressure, then stored until used. Aliquots for use may be withdrawn aseptically from this solution, or the residual solution may be reautoclaved after withdrawal of an aliquot (b) A stock solution containing per cc. 1 mg. each of adenine sulfate, guanine hydrochloride, and uracil. The solution may be kept in the refrigerator under toluene for long periods. (c) A stock solution which contains per cc. sufficient of each vitamin for fifty assay tubes. The vitamin to be determined is omitted from this solution, which is kept under toluene in the refrigerator. A fresh solution should be prepared at intervals not longer than 1 month.

To prepare, for example, the medium for fifty assay tubes one mixes 250 cc. of solution (a), 5 cc. of solution (b), and 1 cc. of solution (c). The addition of 10 gm. of glucose completes the medium, which is twice the concentration given in Table I.

Stock Culture and Inoculum—*Lactobacillus casei* was carried as a stab culture in medium containing 1 per cent Difco yeast extract, 1 per cent glucose, and 2 per cent agar. Stabs were incubated at 37° for 24 to 48 hours, then held in the refrigerator. Stock cultures were transferred at 2 week intervals; stabs for daily use were prepared at the same time as needed.

Inoculum tubes were prepared by diluting the complete double strength medium with an equal volume of water and dispensing this medium in 10 cc. quantities to test-tubes of a size suitable for direct use in the centrifuge. These were plugged with cotton, sterilized, and stored until used. To prepare inoculum a transfer from a stab culture to a tube of inoculum medium was made. This was incubated at 37° for 16 to 24 hours before use.

Assay Procedure—The procedure is similar to that commonly followed at present (*e.g.* (1, 2)) and need not be given in detail. Assays are carried out in 20 × 150 mm. lipless test-tubes. Suitable aliquots of the standard and sample solutions are added to the assay tubes and the volume adjusted with water to 5 cc. 5 cc. of the basal medium (double strength) are then

added to each tube.   The tubes are plugged with cotton or covered with clean aluminum caps and sterilized in the autoclave at 15 pounds pressure for 10 minutes.   More prolonged autoclaving should be avoided, since it partially caramelizes the sugar and produces a dark colored medium with inferior growth-promoting properties for *Lactobacillus casei*.   After cooling to room temperature, they are ready for inoculation.

The cells in a tube of inoculum, prepared as described above, are thrown down by centrifugation, and the supernatant liquid is removed aseptically and replaced by 10 cc. of a sterile solution of sodium chloride (0.86 per cent). The cells are resuspended by shaking.   A sufficient amount of this heavy suspension is added to a second 10 cc. portion of salt solution to give a suspension which is just visibly turbid.   1 drop of this latter suspension is added aseptically to each assay tube.   These are incubated at 37° for 16 hours, if the turbidimetric method is used, or for 72 hours, if the acidimetric method is used.

Measurement of Response—The medium and method described herein were designed especially for the rapid turbidimetric determination of vitamins, particularly riboflavin and folic acid.   Turbidity on the above medium has reached a maximum in 16 to 18 hours.   At the end of this period the sedimented organisms are thoroughly suspended by shaking, and turbidity comparisons are made.   Any reliable photoelectric colorimeter may be used for this purpose; an uninoculated tube of medium serves as the blank.   The measurements are best made at a wave-length at which the medium shows little or no absorption (*e.g.* 660 mμ).   For the purpose of this paper, a calibration curve, relating galvanometer readings of the instrument used to the dry weight of cells obtained, was constructed, and turbidity measurements are given in terms of the dry weight of cells.   For routine use, this procedure is unnecessary; galvanometer readings or optical densities may be used directly.

Acid production continues for considerable periods after turbidity has reached a maximum; the customary 3 day period of incubation was therefore used when titrations were made.   Titration with brom-thymol blue as visual indicator is unsatisfactory because of the high buffer capacity of the medium.   Electrometric titration to pH 6.8, with the assembly described by McQuarrie and Konen (11), was used with complete satisfaction.   The addition of brom-thymol blue permits a rapid visual approach to the endpoint, which is then determined exactly with the pH meter.

Response to Pure Vitamins—Growth response of *Lactobacillus casei* to the addition of the pure vitamins, as was determined turbidimetrically at the end of a 16 hour incubation period, is shown in Figs. 1 to 5.   The enhanced growth-promoting powers of the new medium are emphasized by the comparisons with other widely used media for assay of riboflavin and

folic acid given in Figs. 1 and 2. Acidimetric comparisons of these same media after 3 day incubation periods are given in Table II. With the longer incubation period, the superiority of the new medium, although still evident, is less marked. The great apparent superiority in acidimetric
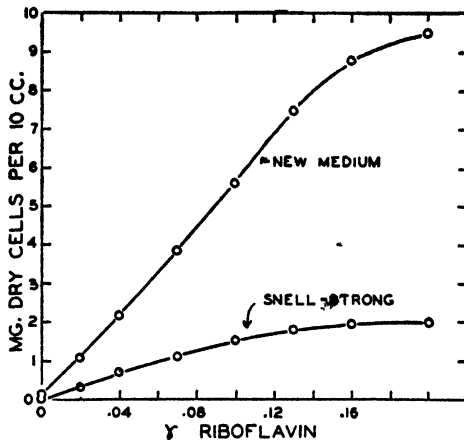
FIG. 1. Comparative turbidimetric response of *Lactobacillus casei* to riboflavin n two media after 16 hours incubation.
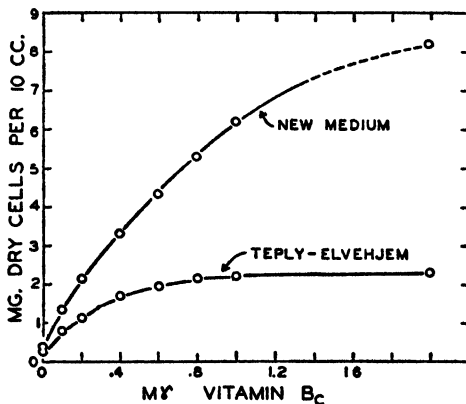
FIG. 2. Comparative turbidimetric response of *Lactobacillus casei* to vitamin B₍ (folic acid) in two media after 16 hours incubation.

response to riboflavin on the new medium is partially deceptive, since it contains twice as much sugar and has over 3 times the buffer capacity of the older medium (*cf.* (12)). Acidimetric response to nicotinic acid, pantothenic acid, and biotin on the new medium is shown in Table III. Acid production per unit of added vitamin is superior in every case to that noted with previous media.

It is probable that the high "blanks" noted in the response to biotin and to pantothenic acid could be lowered considerably by growing the cultures used for inoculum in the basal medium, supplemented with only a minimum amount of the vitamin in question.
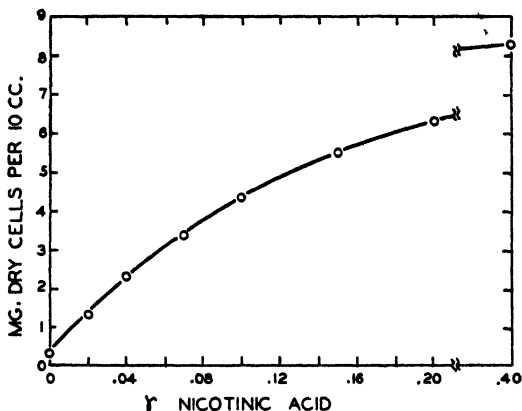
FIG. 3. Turbidimetric response of *Lactobacillus casei* to nicotinic acid in the new medium (16 hours).
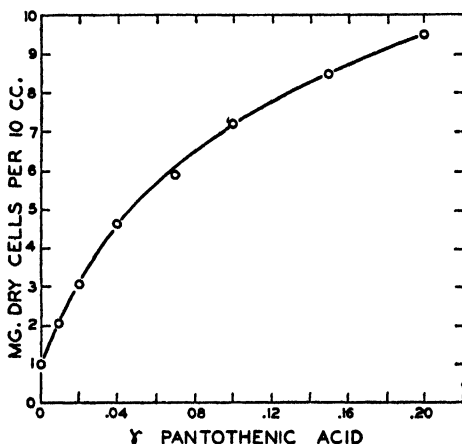
FIG. 4. Turbidimetric response of *Lactobacillus casei* to pantothenic acid in the new medium (16 hours).

Application to Determination of Riboflavin and Folic Acid—To test the applicability of the new medium, a number of natural materials were extracted with acid by a standard procedure (13) and the extracts assayed for riboflavin. Parallel assays were made by the turbidimetric (16 hours) and acidimetric (72 hours) procedures, and the values obtained compared with those obtained on the same extracts by the method of Snell and Strong

(1). Essentially identical results were obtained with each procedure (Table IV); quantitative recoveries of added riboflavin were also obtained.

No rigorously tested or widely accepted method for quantitative extraction or determination of folic acid is yet available. For purposes of com-
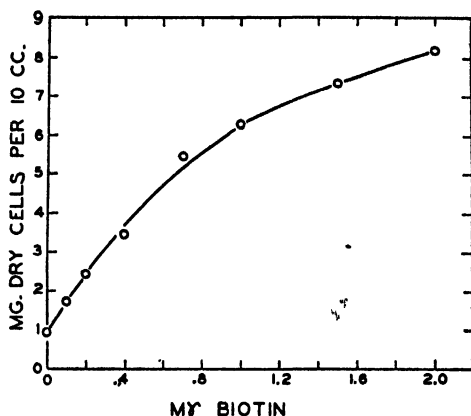
FIG. 5. Turbidimetric response of *Lactobacillus casei* to biotin in the new medium (16 hours).

TABLE II

Comparative Acid Production in Response to Added Riboflavin and Folic Acid on New and Older Media

Titrated after 72 hours incubation at 37°.

Riboflavin added	0.1 N acid produced		Vitamin B_c added	0.1 N acid produced	
	New medium	Snell-Strong		New medium	Teply-Elvehjem
γ per 10 cc.	cc.	cc.	millimicrograms per 10 cc.	cc.	cc.
0	0.30	0.50	0	1.7	2.2
0.02	4.0	1.7	0.10	5.0	4.6
0.04	6.2	2.8	0.20	7.2	6.8
0.07	8.3	4.4	0.40	10.5	9.9
0.10	10.0	6.2	0.60	12.8	12.4
0.13	11.5	7.8	0.80	14.1	13.7
0.16	12.9	9.0	1.0	15.0	14.6
0.20	14.3	9.8	2.0	16.7	16.7

parison, the method of Teply and Elvehjem (6) and an extraction procedure involving digestion with taka-diastase and papain (14) were employed. It is recognized that the latter procedure does not give maximum values for the folic acid content of many natural materials. A preparation of crystalline vitamin B_c obtained from Parke, Davis and Company served as a standard. The results (Table V) were essentially

identical with all of the procedures employed.   With alfalfa, a pronounced drift in values with increasing level of the sample was obtained in both

TABLE III

Titrimetric Response of Lactobacillus casei to Nicotinic Acid, Pantothenic Acid, and Biotin in New Medium

Titrated after 72 hours incubation at 37°.

Nicotinic acid		Pantothenic acid		Biotin	
Amount added	0.1 N acid produced	Amount added	0.1 N acid produced	Amount added	0.1 N acid produced
γ per 10 cc.	*cc.*	*γ per 10 cc.*	*cc.*	*millimicrograms per 10 cc.*	*cc.*
0	0.8	0	2.4	0	3.0
0.02	3.4	0.01	4.5	0.1	5.2
0.04	6.0	0.02	6.2	0.2	6.5
0.07	8.1	0.04	8.6	0.4	8.4
0.10	10.0	.0.07	10.7	0.7	10.2
0.15	11.5	0.10	12.6	1.0	11.1
0.20	12.0	0.15	13.5	1.5	12.2
0.40	13.9	0.20	13.8	2.0	13.0

TABLE IV

Comparative Determinations and Recoveries of Riboflavin

Samples	Riboflavin found			Recoveries; present method	
	Present method*		Snell-Strong, acidimetric (72 hrs.)	Turbidi-metric (16 hrs.)	Acidimetric (72 hrs.)
	Turbidi-metric (16 hrs.)	Acidimetric (72 hrs.)			
	γ per gm.	*γ per gm.*	*γ per gm.*	*per cent*	*per cent*
Yeast extract (Bacto).......	44.2	42.4	43.8	109	106
Liver concentrate (Wilson's No. 1:20)..................	186	182	193	103	107
Bacto-peptone..............	3.3	2.9	3.5	97	102
American cheese............	6.3	6.4	5.9	100	108
Milk (fresh)................	1.8	1.8	1.8	103	109
Skim milk powder...........	19.8	21.4	20.0	100	95
Rat kidney.................	25	22	27	95	105
" liver....................	18	19	19	92	100
" muscle.................	1.3	1.5	1.5	107	107
Canned peas................	0.44	0.48	0.52	91	102

* Assay range, 0.02 to 0.15 γ of riboflavin.

acidimetric assays; this was not apparent in the shorter turbidimetric procedure.   Quantitative recoveries of added vitamin B₆ were obtained.

Effect of Fatty Substances—The disturbing influence of fatty acids, lecithins, etc., on the response of *Lactobacillus casei* to suboptimal amounts of riboflavin and other vitamins has been noted elsewhere (15–17) and studied in detail by Kodicek and Worden (18). The same effects are observed with the new medium. Lecithin, for example, greatly stimulates the response to riboflavin at low levels but suppresses it somewhat at high levels. The addition of 50 γ of oleic acid per 10 cc. of medium suppresses

TABLE V

Comparative Determinations and Recoveries of Folic Acid

Samples	Folic acid found			Recoveries; present method	
	Present method*		Teply-Elvehjem, acidimetric (72 hrs.)	Turbidi-metric (16 hrs.)	Acidimetric (72 hrs.)
	Turbidi-metric (16 hrs.)	Acidimetric (72 hrs.)			
	γ *per gm.*	γ *per gm.*	γ *per gm.*	*per cent*	*per cent*
Whole wheat flour...........	0.32	0.35	0.33	97	97
White flour.................	0.31	0.35	0.35	107	100
Liver concentrate...........	20	21	21	107	95
Beef muscle.................	0.44	0.46	0.48	104	102
Potatoes (fresh)............	0.46	0.48	0.48	102	98
Tomatoes "	0.36	0.37	0.35	98	105
Alfalfa.....................	1.2	0.5–2.5	0.6–3.3	95	

* Assay range, 0.1 to 1.0 millimicrogram of folic acid per 10 cc. of medium.

growth completely; the simultaneous addition of 100 γ of lecithin completely overcomes this inhibitory effect. Measures such as solvent extraction and filtration at pH 4.5, which are already in use to prevent these effects in other media, appear adequate with the present medium as well.

DISCUSSION

Recent years have seen a great expansion in the development and use of microbiological assay methods. As experience with the assay organisms accumulated, it was inevitable that improvements in the early methods should result. In many cases the sole criterion used to determine whether modification of an assay medium has resulted in improvement has been to determine whether increased growth or acid production resulted from the modification. Judged by this standard, the present medium is greatly improved. over former media used with *Lactobacillus casei*. It does not necessarily follow, however, that a medium which permits greater growth and acid production will necessarily permit more accurate bioassays. Examination of Tables IV and V reveals in fact that the present medium,

although permitting accurate assays with increased growth and acid production, gives the same values as previously proposed assay methods. The modification is valuable because it permits greatly shortened assays to be carried out; assays inoculated at the close of 1 working day are ready to be read at the beginning of the next. If the 3 day acidimetric method is used, the new procedure appears to have little or no advantage over older methods.

The proposed procedure is also advantageous in that a single basal medium and assay organism suffice for assay of a number of vitamins. Routinely, the preparation of enzymatic digests of casein is considerably less troublesome than that of acid hydrolysates of casein.

All ingredients of the culture medium are present in considerable excess; dilution of the complete medium with an equal volume of water gives a medium which supports growth only slightly less luxuriantly. Although supplementation of the medium with natural materials has not been necessary to obtain accurate values with materials so far assayed, addition of such supplements may prove valuable for special purposes. Supplements free of riboflavin are readily obtained by photolysis (19, 20) while natural extracts treated with lead acetate in the same manner used in the preparation of the yeast supplement for riboflavin assay (1) are free of both riboflavin and folic acid.

Other commonly used assay organisms, such as *Lactobacillus arabinosus* and *Lactobacillus pentosus*, also produce heavy growth in the new medium during a 16 hour incubation period. The medium could, therefore, be used with either of these organisms for the rapid turbidimetric determination of nicotinic acid, pantothenic acid, and biotin. Results obtained in this manner have not been compared with those given by previously published procedures.

SUMMARY

A medium is described which permits the accurate turbidimetric determination of riboflavin and folic acid with *Lactobacillus casei* after a 16 hour incubation period. Excellent growth curves are also obtained with nicotinic acid, pantothenic acid, and biotin, although use of the medium for determination of these vitamins has not been investigated.

Results obtained turbidimetrically at 16 hours check those obtained acidimetrically at 72 hours; they also check those obtained by previously published procedures.

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DETERMINATION OF POLYSACCHARIDE IN SERUM

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That there exists a true polysaccharide in the serum in close association with the serum proteins has been shown by a number of investigations including those of Bierry (1), Everett and Sheppard (2), Rimington (3), Lustig *et al.* (4), Hewitt (5), Nilsson (6), and Blix, Tiselius, and Svensson (7). In spite of the fact that there is a considerable concentration present in the serum, as great or greater than of glucose, comparatively few attempts have been made to evaluate it quantitatively with a view to determining its significance. This may be due in part to the fact that its quantitative estimation has been troublesome and in many cases inaccurate.

The present paper aims to present a quantitative method for its determination, which is accurate, reproducible, and reliable. It is nevertheless tedious in that strict adherence to the details of the method and only very careful technique will yield desirable results. If such care is employed, the method is dependable, as is shown by the data.

Everett and Sheppard (2) applied the colorimetric methods of Folin and Wu, Benedict, Sumner, and Folin to hydrolysates of blood filtrate. Lustig (4) and collaborators determined the carbohydrate directly by the Tillmans-Philippi orcinol reagent, and Nilsson (6) determined the carbohydrate as glucosamine. The present study utilizes the carbazole reagent of Dische (8). The color produced varies from a deep pink for glucose and related sugars to a brownish red for mannose and can thus be used in differentiating the type of carbohydrate accompanying the proteins under investigation.

Techniques

Total Carbohydrate

The total carbohydrate content of serum is determined by use of the carbazole reaction of Dische (8), as stated previously, and for the comparison of colors, first (9) the Duboscq, then (10) the Evelyn, and now the Klett colorimeter, with confirmation by the Beckman spectrophotometer.

Any of these instruments, as well as others, should be adaptable in this method, but for quick routine work we have found the Klett instrument very useful. In the region of absorption most used in the method as outlined, four scale divisions on the Klett instrument are equivalent to one division on the Evelyn, and this is the largest variation in readings between duplicate determinations which has been accepted by us (most readings vary only one or two divisions). This corresponds to a final difference of 2 to 4 mg. per cent in the serum, with a maximum of 8 mg. per cent, in the method as used.

Reagents—

1. Sulfuric acid reagent, 1 part of distilled water plus 8 parts of concentrated sulfuric acid (Baker's analyzed sulfuric acid, C.P. special, low in N and As).

2. Saline, 0.9 per cent.

3. Carbazole reagent, 0.5 per cent in absolute alcohol. Eastman Kodak carbazole is purified by twice subliming it. If sufficiently pure, it will remain colorless indefinitely when dissolved in the alcohol and kept in a glass-stoppered bottle. It should be decanted free from any trace of sediment before use.

Standard (G. G. M.)—Equal amounts each of 0.01 per cent glucose, galactose, and mannose (purest grades) are mixed.

This standard is used in analyzing whole serum, since Hewitt (5) agreed with previous authors that the carbohydrate group in serum proteins is a polysaccharide containing equimolecular amounts of galactose, mannose, and glucosamine.

Furthermore, the absorption curve given by serum with carbazole, as well as the shade of color observed, corresponds closely with that of the G. G. M. standard described below. Pure glucose alone gives a more rose, while mannose alone gives a brownish red color.

In order to determine the proper wave-length to be used for routine purposes, readings were made in a Beckman spectrophotometer, at all wave-lengths, of the color produced by carbazole with 0.1 mg. of the isolated sugars or of the mixture of sugars. Similar readings at all wave-lengths were also made of the blank solutions of the same sugars, treated in the same manner but without the carbazole, and these results were subtracted from the above corresponding values, giving the curves (Fig. 1) which represent the true absorption curves of the sugars. They show that there are two wave-lengths at which definite absorption occurs; namely, at 5400 and at 4400 A. It is furthermore clear that the relative amounts of absorption at these two wave-lengths differ markedly with the different sugars and that a ratio of their extinction or density values, log (1/transmission), at these two wave-lengths would be characteristic of each sugar, as was also shown

by Gurin and Hood (11). Concentrations of different carbohydrates were therefore determined in the usual photoelectric colorimeters with the use of a filter giving the maximum absorption at wave-length 5400 A. Since identification of the type of carbohydrate present in a mixture can be made by determining the ratio D at 5400 A to D at 4400 A, and since the ratio found for serum corresponds more closely with that of the G. G. M. mixture, we have, therefore, used the latter as the standard for evaluating the polysaccharide of serum. The ratios found for the sugars of significance in this study were as shown in Table I.
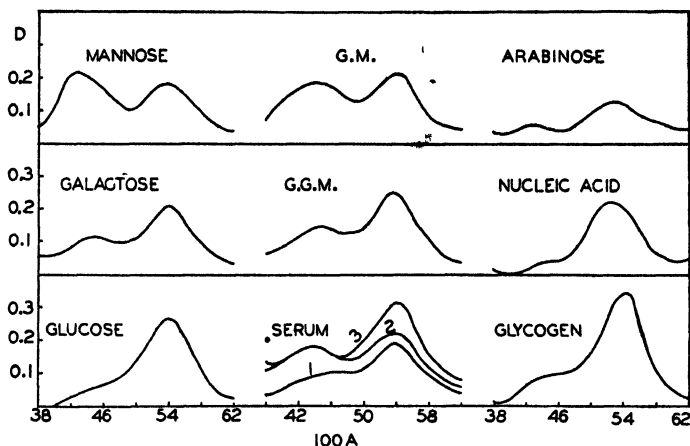
FIG. 1. Density curves of various sugars and sugar mixtures. G. M. represents equal concentrations of galactose and mannose; G. G. M., equal concentrations of galactose, mannose, and glucose; Serum 1, 2, and 3, curves for samples from one individual obtained by alcohol precipitation, and from another during fasting, and following a meal respectively.

Fig. 2 shows that the intensity of color produced at wave-length 5400 A by different concentrations of the single sugars as well as of the mixtures followed Beer's law.

Method—Serum is diluted so that 0.5 ml. is made to 10 ml. with 0.9 per cent saline. Each ml. is equal to 0.05 ml. of original serum.

To fourteen tubes (25 × 150 mm.) are added 1 ml. of distilled water and 8 ml. of concentrated sulfuric acid or 9 ml. of the sulfuric acid reagent. These are shaken and chilled in an ice bath, and then to six tubes, three each for duplicate dilutions of the serum, 1 ml. of the diluted serum is carefully layered on the top surface of the acid, *without wetting the side of the tube*. To each of four control tubes 1 ml. of distilled water is added. To each of four standard tubes 1 ml. of the standard solution is added. All tubes are again shaken, preferably under ice, care being taken that all the protein is

completely dissolved.   Then 0.3 ml. of the 0.5 per cent carbazole is added to four of the sera tubes (two from each dilution, Tubes A and B), to two

TABLE I

Density Ratios

Sample, 0.1 mg.	D at 5400 A	D at 4400 A	$\frac{D \text{ at } 5400 \text{ A}}{D \text{ at } 4400 \text{ A}}$
Arabinose........................	0.125	0.059	2.12
Nucleic acid (thymus).............	0.185	0.038	4.87
Glycogen.........................	0.342	0.075	4.56
Glucose..........................	0.274	0.056	4.89
Galactose........................	0.196	0.103	1.90
Mannose.........................	0.176	0.218	0.81
Galactose-mannose...............	0.210	0.190	1.11
Glucose-galactose-mannose........	0.243	0.143	1.70
Serum (normal), fasting...........	0.223	0.157	1.42
" " after high carbo-hydrate........................	0.316	0.176	1.79
Serum-alcohol ppt., normal.......	0.209	0.097	2.15
" " tuberculous...	0.263	0.117	2.24
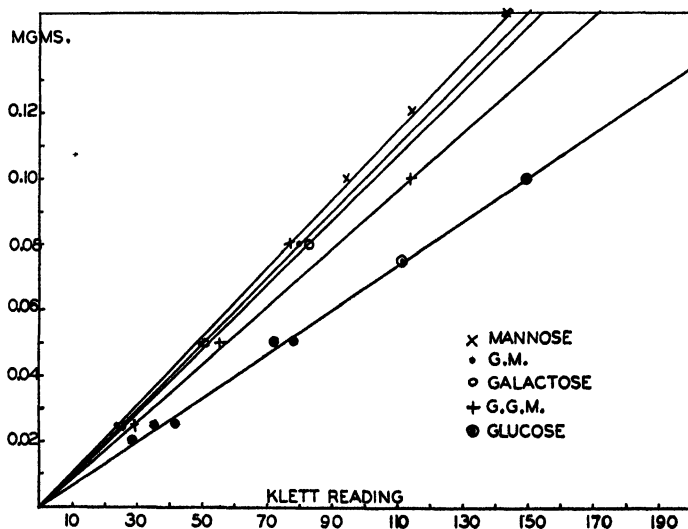
FIG. 2. Readings on Klett colorimeter plotted against the concentration of sugars used in the carbazole reaction, showing that Beer's law is satisfied.

standard tubes (C), and to two control tubes (D).   To the two remaining sera tubes (A' and B'), the two remaining standard tubes (C'), and the two remaining control tubes (D'), 0.3 ml. of absolute alcohol (with no carbazole)

is added. All are again shaken, covered with glass bottle stoppers, and placed simultaneously in a vigorously boiling water bath for exactly 10 minutes. They are then simultaneously removed and cooled in a chilled water bath, and then read in a colorimeter at wave-length 5400 A.

Tubes D′ are read against each other and should check.

Tubes D are read against Tube D′ to determine the amount of color given by the carbazole. This should be low and gives an indication of the purity of the carbazole and acid used.

All tubes are read against a Tube D′.

Calculation—The serum blank and carbazole blank readings are subtracted from the serum readings. The G. G. M. blank and carbazole blank readings are subtracted from the G. G. M. readings. Since a slight difference may occur in the intensity of the color, produced by the standard, possibly due to heating of the reagent, the standard is always run with every set of analyses and, therefore, by calculation any variation in the results due to such small errors may be eliminated. Thus the reading due to 0.1 mg. of the standard divided into that due to 1 ml. of the unknown, multiplied by 10, will give the amount of total carbohydrate in 1 ml. of the dilution of the unknown.

Glucose

Free glucose in the serum was determined by means of the Somogyi (12) modification of the Shaffer-Hartmann method, using the $ZnSO_4$-$Ba(OH)_2$ reagent for deproteinizing the serum, as outlined by Nelson (13) prior to publication by Somogyi (14).

Polysaccharide of Serum

The total carbohydrate of the serum minus the glucose gives the true polysaccharide. Moreover, this polysaccharide can be determined directly, since it is either precipitated by alcohol or carried down with the serum proteins when they are precipitated with alcohol.

Method for Direct Determination of Polysaccharide—0.1 ml. of serum is dropped into 10 ml. of 95 per cent alcohol with shaking and with care not to run the serum down the side of the tube. With this technique the precipitate usually flocculates out in such a way that it is easy to centrifuge for 30 minutes, leaving a crystal clear supernatant. Other procedures, such as those used by Lustig and by Langer (4), or Lustig and Nassau (15), yielded cloudy supernatants in our hands with resultant loss of protein.

The precipitate is then washed with 5 ml. more of alcohol and centrifuged. The supernatants are carefully sucked off without disturbing the precipitate. The precipitate is then immersed in an ice bath to prevent charring when the acid is added. Since occasionally a serum precipitates as

a gum instead of in floccules, 4 ml. of cold concentrated sulfuric acid are carefully added directly to the precipitate in order to effect solution. When shaken and cooled, 1.9 ml. of water are added, and, again when cool, 4 ml. more of cold concentrated H_2SO_4 are added.' Four such tubes are prepared and to two of them as controls is added 0.3 ml. of alcohol, while to the other two 0.3 ml. of carbazole reagent is added. These are shaken, covered with glass stoppers, and heated in a boiling water bath for exactly 10 minutes, then chilled, and read in the colorimeter. Simultaneously water blanks, carbazole blanks, and tubes with the standard and its blanks are also run as described above under total carbohydrate determination. The final calculation is also the same.

In this case, however, a standard consisting of galactose alone is used. It was at first believed that a standard of galactose and mannose (G. M.) should be used, since the glucose of the serum had been removed by the

. TABLE II

Recovery of Glucose (100.0 Mg. Per Cent Added) As Glucose in Serum

Subject	Glucose in original serum	Glucose found	Glucose recovered	Error
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
J. H.	99.1	196.6	96.6	−3.4
J. B.	85.3	186.9	101.6	+1.6

precipitation, but an absorption curve (see Fig. 1) for the alcohol precipitate of serum indicated a type of absorption more closely corresponding to that of galactose alone than of a mixture of galactose and mannose (G. M.). This conclusion is supported by the ratios recorded in Table I. The explanation for this result may throw some light on the composition of the carbohydrate present in the serum and must wait for future consideration.

EXPERIMENTAL

Recovery of Glucose Added—In order to study the reliability of the methods chosen an attempt was made to determine the recovery of glucose as glucose and as total carbohydrate when added to serum. For example, Table II shows that, when a known amount of glucose was added to two sera, copper iodometric titration analyses of the glucose on the original sera and on those after the addition varied to the extent of the amount of glucose added, within experimental error.

When glucose is added to serum, it can also be recovered in the total carbohydrate, determined by means of the carbazole reaction and read against the G. G. M. standard. The reading on the Klett colorimeter for

the original serum is subtracted from the reading for the same dilution of serum containing the added glucose and this result is then converted into its equivalent of the G. G. M. standard. The result corresponds to the added glucose (see Table III).

Check on True Polysaccharide Concentration—Table IV shows the close correspondence between the concentration of true polysaccharide determined by difference and directly on the same sera, indicating that the

TABLE III

Recovery of Glucose in Total Carbohydrate

Subject	Klett reading (5400 A)			Carbohydrate equivalent as glucose-galactose-mannose	Glucose added	Error
	Serum + glucose	Serum + saline	Difference			
				mg. per ml.	*mg. per ml.*	*mg. per ml.*
J. H.	82.3	42.7	39.6	0.0355	0.0380	−0.0025
J. B.	82.3	37.3	45.0	0.0405	0.0380	+0.0025
H. G.	96.7	55.0	41.7	0.0370	0.0380	−0.0010
J. A.	85.0	43.3	41.7	0.0370	0.0380	−0.0010
A. B.	82.3	38.3	44.0	0.0400	0.0380	+0.0020

TABLE IV

True Polysaccharide by Difference and by Direct Analysis

Subject	Total carbohydrate	Glucose	True polysaccharide by difference	True polysaccharide by direct determination
	mg. per cent	*mg. per cent*	*mg. per cent*	*mg. per cent*
M. D.	201.6	74.8	126.8	118.7
E. P.	212.5	49.0	163.5	165.8
E. H.	212.0	96.6	115.4	113.5
V. P.	195.8	89.8	106.0	102.4
S. B.	177.1	63.3	113.8	106.8
W. McC.	273.8	96.6	177.2	172.1
J. H.	276.4	168.0	108.4	107.4
E. Sch.	193.2	88.4	104.8	100.5

techniques chosen for the determination of total carbohydrate and for the alcohol-precipitable carbohydrate give essentially identical results.

Keeping Qualities of Sera When Frozen—If sera are frozen several hours after the bloods are taken, no significant changes, greater than can be accounted for by experimental error, occur in their content of total carbohydrate or glucose over indefinite periods of time. This is shown in Tables V and VI. If kept in this manner, therefore, it is possible to perform the analyses at the time most convenient and economical from the standpoint of the laboratory schedule.

Variation in Polysaccharide from Time to Time—It is of importance to know also the spread in the polysaccharide value of sera taken from the

TABLE V

Variation of Glucose in Sera Kept Frozen

Subject	Date bled	Date of run	Glucose
	1945	*1945*	*mg. per cent*
J. H.	Apr. 11	Apr. 16	101.4
		July 18	96.1
		" 31	99.1
J. A.	" 27	May 1	103.0
		June 4	101.9
" .	July 5	July 9	87.1
		" 17	89.4
M. H.	June 8	June 8	119.0
		" 15	123.2
N. H.	July 16	Oct. 9	85.4
		" 17	85.0

TABLE VI

Variation of Total Carbohydrate in Sera Kept Frozen

Subject	Date bled	Date of run	Total carbohydrate
	1945	*1945*	*mg. per cent*
J. H. .	Apr. 11	Apr. 16	160.7
		July 18	156.8
		Aug. 21	162.0
J. A.	" 27	May 1	173.2
		June 4	168.8
		Aug. 22	182.4
J. B.	June 8	June 12	157.6
		Aug. 1	152.4
H. G.	July 13	July 20	234.2
		Aug. 17	228.6
A. B.	June 19	July 16	164.2
		Aug. 23	168.2
		Oct. 31	174.6

same non-fasting individual at different times and, therefore, a number of such results are included in Table VII.

Variation in Polysaccharide Due to Metabolism—Since the variation in the true polysaccharide concentration of the serum in the same individual from time to time appears to be considerable, as much as 27 mg. per cent in one case, it seemed advisable to determine more directly the effect of metabo-

lism on this constituent. Therefore, the following experiments were de-
signed to study this aspect.

Blood was taken from fasting subject J. A. and then the following
meal was consumed: ½ cup of whole wheat cereal, 3 gm. of agar-agar, 7 gm.

TABLE VII

Variation in Serum Polysaccharide of Same Individuals at Different Times

Subject	Date bled	Total carbohydrate	Glucose	True polysaccharide
	1945	*mg. per cent*	*mg. per cent*	*mg. per cent*
J. A.	Apr. 27	173.2	100.3	72.9
	July 5	171.4	78.2	93.2
	" 16	132.8	38.8	94.0
F. S.	Apr. 27	276.0	168.0	108.0
	July 5	193.4	92.5	100.9
	Sept. 9	186.3	96.6	89.7
	Dec. 4	196.2	88.4	107.8
G. D. (advanced tuber-	Feb. 5	258.2	102.0	156.2
culosis)	May 3	250.5	84.3	166.2
L. D. (minimal tuberculosis)	Feb. 8	143.0	57.8	85.2
	July 19	168.4	78.2	90.2
	Oct. 29	185.1	88.4	96.7
E. S. (sarcoidosis)	Apr. 11	204.5	88.4	116.1
	Nov. 14	219.6	76.2	143.4

TABLE VIII

Effect of Metabolism on Polysaccharide Concentration

Subject	Time of bleeding	Total carbohydrate	Glucose	True polysaccharide
		mg. per cent	*mg. per cent*	*mg. per cent*
J. A.	Fasting	151.3	71.4	79.9
	After meal, 3 hrs.	171.4	78.2	93.2
J. H.	Fasting	204.0	82.3	121.7
	After meal, 30 min.	276.4	168.0	108.4
F. S.	Fasting	186.0	85.0	101.0
	After meal, 30 min.	184.2	88.4	95.8
	" " 2 hrs.	196.2	88.4	107.8

of potato starch, ½ teaspoon of sugar, 1 pint of milk, 20 cherries, 2 small
oranges and the pulp from these oranges. 3 hours later a second blood
sample was taken. This diet was planned to contain galactan, galactose,
starch, sucrose, and arabinose from the cherries and orange pulp pectin.
The results are given in Table VIII.

Blood was taken from fasting subject J. H. and then the following meal was consumed: 1 cheese and ham sandwich on rye bread, 1 slice of white bread, 1 egg, 1 orange, a piece of pickle, 2 crackers with jelly, 1 orange, 1 glass of milk, 3 cups of tea, 100 gm. of maltose. ½ hour after the meal a second blood sample was taken.

Blood was taken from fasting subject F. S. and then the following meal was consumed: 1 pint of milk, 2 oranges, 2 pieces of Swedish bread (about 100 gm.) with much butter, 24 dried brewers' yeast tablets (= 7.0 gm. of dried yeast) as a source of mannose. ½ hour and 2 hours after the meal additional blood samples were taken.

These experiments show that relatively little change occurs in the true polysaccharide content of serum due directly to the ingestion of food, even when the glucose content (see J. H. in Table VIII) doubles. The largest change which occurred in three experiments was 13.3 mg. This is not as much as twice the experimental error of the method.

Variation in Normal Individuals—The concentration of true polysaccharide in the non-fasting sera of thirty-nine normal individuals was found to vary from 72.9 to 131.0 mg. per cent, with an average of 102.2 mg. per cent. Variations found in different pathological conditions will be given in another paper.

DISCUSSION

There are certain errors automatically inherent in the method, which should be understood. For example, it is clear that glucose gives more color per unit with the carbazole reagent than does mannose or galactose. Consequently, of sera checked against the same standard, those containing a high glucose content will appear to contain falsely high polysaccharide concentrations, while those with low glucose will be more nearly correct. Therefore, theoretically a different standard, containing varying amounts of glucose, should be used for each serum, depending upon the amount of glucose present. This, of course, would be impractical, even if possible. and the conclusion naturally arises that it would be better to employ a method in which the glucose is eliminated from the serum before the analysis. Such a method is that described in this paper as the "direct determination of polysaccharide." This method, furthermore, decreases the experimental error by eliminating one step in the analysis.

As mentioned in the description of the method, a standard is run with each set of determinations and the value obtained on this standard is used in the calculation of the results for all other products analyzed at the same time. This is done because the reading for the standard will fluctuate from time to time as much as ±5 around an average value. Since it is very

desirable to eliminate this fluctuation, a careful investigation was made as to its cause.

It was found, for example, that much of the difficulty lay with the carbazole reagent itself, and attempts were made to overcome it by dissolving the carbazole in concentrated sulfuric acid rather than in alcohol. Such a reagent gave results which checked very well, all of which were considerably higher than those obtained with the alcoholic carbazole. However, a fluctuation still occurred in the readings for the standard sugar solutions and, since this acid carbazole reagent must be made fresh each day, there seemed to be no advantage in its use.

During these experiments it was found that alcohol decreases the color produced but since the same amount of alcohol is added to all tubes no error is caused in this way. It is interesting, however, that the decrease in color by the alcohol is caused in the sugar blanks, containing only the sugars and sulfuric acid, as well as in the tubes containing carbazole.

In view of the fact that no solution for the elimination of the fluctuation has so far been found, the method should be used exactly as outlined in order to obtain reproducible results.

SUMMARY

A colorimetric method based upon the carbazole reaction is outlined for the quantitative determination of the polysaccharide content of serum.

Absorption maxima of the compound formed between carbohydrate and carbazole occurred at two wave-lengths (5400 and 4400 A). Since the relative amounts of these absorptions varied for different types of sugars, the proper standard could be chosen for comparison with the carbohydrate under investigation, even if unknown, based upon the ratio of the extinction coefficients at these two wave-lengths.

Comparable results were obtained whether the determination was made directly upon the polysaccharide which precipitated with the serum proteins in alcohol, or by subtracting the glucose content of serum from the total carbohydrate concentration.

The concentration of carbohydrate in serum including glucose did not change over an indefinite period of time, if the sera were kept in the frozen state or just at the point of freezing.

The average serum concentration of polysaccharide in thirty-nine normal individuals was 102.2 mg. per cent, with a range from 72.9 to 131.0 mg. per cent and the same individual bled at different times did not show much change unless disease was present.

In three subjects a maximum variation of 13.3 mg. per cent of polysaccharide was noted between the concentrations during fasting and after ingestion of meals containing considerable amounts of carbohydrate.

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METABOLISM OF THE MALARIAL PARASITE, WITH REFERENCE PARTICULARLY TO THE ACTION OF ANTIMALARIAL AGENTS

I. PREPARATION AND PROPERTIES OF PLASMODIUM LOPHURAE SEPARATED FROM THE RED CELLS OF DUCK BLOOD BY MEANS OF SAPONIN*

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A study of certain aspects of the metabolism of the malarial parasite, *Plasmodium lophurae*, from duck blood was undertaken in an attempt to elucidate the mode of action of some of the antimalarial drugs. It had already been shown by Christophers and Fulton (1) that parasitized monkey blood exhibited a rapid oxygen consumption in the presence of glucose, and that this was suppressed by quinine (2), atabrine, plasmochin, and related drugs. Similar effects have subsequently been noted by others in observations with *P. gallinaceum* in the red cells of chicken blood as well as with *P. knowlesi* (3, 4). However, the nature of the inhibition and whether it was primary or secondary to a lethal action on the parasites were not indicated.

It was believed that a clearer picture of the metabolism of the parasites might be obtained with parasites separated from the red blood cells, especially in the case of those that occur in nucleated red cells. Christophers and Fulton found that *Plasmodium knowlesi*, separated from the red cells of monkey blood by saponin, was capable of some respiration in the presence of glucose (5). More recently Speck and Evans by a similar method separated *P. gallinaceum* from the red cells of chicken blood and investigated some of the individual enzymes found in parasite extracts with regard to their sensitivity to atabrine and quinine (6). When attempts were made to separate *P. lophurae* from the red cells of duck blood by either of the described methods, it was found that the parasites had only a very low and rather rapidly declining rate of oxygen uptake, as compared to the stable respiration of the parasitized red cells. Therefore some alterations in the method of preparation to avoid damage to the parasites were necessary before material of high and moderately stable oxygen up-

take could be obtained. The method finally adopted for destruction of the red cells, as well as some of the properties of the separated parasites thereby prepared, is described below.

Methods

Oxygen consumption was measured by the usual Warburg technique at 37°. Each flask contained 0.6 ml. of cell suspension diluted to a total volume of 2.4 ml. The buffer-salt solution[1] used in these measurements had the following composition: NaCl 0.0068 M, KCl 0.0865 M, Na_2HPO_4 0.0275 M, KH_2PO_4 0.0025 M, $MgSO_4$ 0.0015 M; pH 7.6 (on addition of cells, the pH at once fell to about 7.3). All solutions added to the reaction mixture were brought to this or a closely similar salt concentration and pH. Glucose, when present, was added to a final concentration of 0.0058 to 0.0116 M.

For washing and suspending parasitized red blood cells and separated parasites, a buffer-salt solution of the following composition was used: NaCl 0.0145 M, KCl 0.088 M, Na_2HPO_4 0.021 M, KH_2PO_4 0.005 M, $NaHCO_3$ 0.004 M, $MgSO_4$ 0.0015 M, glucose 0.0139 M; pH 7.25. The glucose was omitted only for the final washings of cells that were to be used with other substrates.

The parasitized duck blood was obtained through the courtesy of Dr. E. K. Marshall, Jr., and his staff shortly after it had been drawn from a duck with a 3 or 4 day infection of *Plasmodium lophurae*. The total parasite count on different samples varied between 8 and 13 × 10^{8} parasites per ml. The blood was cooled, centrifuged, and the cells washed two or three times with 20 ml. of buffer-glucose solution per 10 ml. of blood, and then resuspended to the original blood volume in the same medium.

After preliminary experiments in which various factors were tested, the following procedure was adopted for the separation of parasites from red blood cells. 1 volume of washed parasitized cells was warmed to 37°; to this was added 0.5 volume of 0.15 per cent saponin, dissolved in the buffer-glucose solution, and the mixture was held at 37° for 15 minutes (or in later experiments, only 5 minutes), followed by dilution with 2.5 volumes of buffer-glucose, and centrifugation at room temperature for 10 minutes. The supernatant was discarded and the parasite-nuclei mixture washed once at room temperature with 4 volumes of buffer-glucose, and then once or twice at 0° with buffer-glucose or buffer alone. The number of washings had no influence on the activity of the parasites. The parasites were finally resuspended in buffer to the original blood volume.

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[1] A similar solution was described by Trager (7).

Results

The oxygen uptake of washed, parasitized red blood cells varied between 18 to 60 microliters of O_2 per 15 minutes per 0.6 ml. This variation showed no correlation with the total parasite count. Differential counts were not made, but it was noted, in agreement with the observation of others (8–10, 4), that the low rates of oxygen uptake were found with blood samples containing predominantly small forms of the parasites, and the higher rates with larger parasites.

In evaluating the suitability of the procedures tried for preparation of cell-free parasites, the washed red blood cells were treated with saponin, washed, and finally resuspended to the initial volume or some known fraction thereof. The rate of O_2 uptake by 0.6 ml. of separated parasites could

TABLE I

Effect of Saponin Concentration and Temperature on Oxygen Uptake of Parasites Separated from Red Blood Cells

Experiment No.	Temperature of saponization	Final concentration of saponin	Final volume relative to cell suspension	Control rate of O_2 uptake per 0.6 ml.	Respiratory activity
	°C.	per cent		microliters per 15 min.	per cent of initial
8-1	0	0.1	2	55	3
	37	0.1	2		13
16-1	Room	0.05	2	38	53
	37	0.05	2		63
10-2	37	0.2	2	42	0
		0.1	2		32
17-1	37	0.1	1.1	50	58
		0.05	2		71
		0.05	1.1		79

then be directly compared with that of 0.6 ml. of the original parasitized cells. The factors of chief importance in the preparation of active separated parasites were found to be the concentration and amount of saponin, and the temperature at which the cells were in contact with saponin. High yields were favored by use of the minimum amount of saponin required for complete hemolysis, and by saponization at 37°, rather than at any lower temperature (Table I). Microscopic examination of both normal and parasitized duck blood indicated that a concentration of 0.05 per cent saponin, in a final volume 1.5 times that of the cell suspension, was necessary to insure complete hemolysis. Later experiments indicated that some blood samples may require less, so that for the best results the minimum concentration required by each particular blood sample should probably have been determined.

Attempts were made further to improve the "yield" of respiratory activity by change in the composition of the buffer or by addition of various substances with or immediately after the saponin. Variations in pH between 7 and 7.6, in the Na:K ratio from 0.7 to 30, or addition of small amounts of Ca, Mn, Zn, Cu, or Fe, had no demonstrable effect. Addition of duck plasma, 1 volume, or of cholesterol (0.2 to 0.3 mg. per ml., kept in suspension by lecithin, 0.3 to 0.7 mg. per ml.) immediately after the saponin also was without effect, although either would have sufficed to prevent the action of saponin upon the red blood cells if added initially.

Properties of Erythrocyte-Free Parasite Preparations—The preparations[2] showed a rate of O_2 uptake that averaged 70 per cent (55 to 92 per cent) that of the parasitized red cells, when prepared from fresh blood. If prepared 24 hours later, the yield was lower, 54 per cent (38 to 67 per cent). Part of the loss of respiratory activity undoubtedly was attributable to destruction of the red cells. Normal duck red cells had an appreciable O_2 uptake, averaging 6 microliters of O_2 per 15 minutes per 0.6 ml., as compared to 18 to 60 microliters for parasitized cells. The O_2 uptake of the normal cells was completely lost after saponization and washing of the nuclei. It is quite possible that the metabolic activities of host and parasite cells are not distinct entities, and it is certainly unlikely that the fraction of the respiration assignable to the red cells as such is identical with the normal red cell respiration. Therefore it would appear to be impossible to estimate with complete accuracy the loss to be expected on separation of parasites from red cells. If the rate observed with normal cells is used as at least a rough estimate and subtracted from the total to give a measure of the respiration due to parasites in the cells, the respiratory activity retained after saponization of fresh blood becomes 88 per cent (68 to 107 per cent). Some of the variation noted may have resulted from the circumstance mentioned above, that certain blood samples actually required less than the selected amount of saponin for hemolysis.

The respiratory quotient of the separated parasites, with glucose as substrate, varied with different blood samples between 0.7 and 1.0, indicating that with some glucose utilization was complete, with others incomplete.

[2] Dr. E. K. Marshall, Jr., and his staff in experiments with the washed separated parasites found in the latter a definite but low degree of infectivity. The parasites were given intravenously in doses of 1200, 300, 240, 60, and 12×10^6 parasites to groups of three or four ducks weighing 100 to 150 gm. Parasites were first detected in blood smears of all twelve of the birds given 60×10^6 or more parasites between the 8th and 10th days after infection. Parasites were seen in blood smears of two of the four ducks receiving 12×10^6 between 10 and 13 days after infection. It was estimated from the day of appearance of the infection that less than 100 *infective* parasites apparently were contributed by the dosage of 12×10^6.

A similar variation in the R.Q. of parasitized red cells (10) has been described.

The respiratory activity of the separated parasites kept at 0° was less stable than that of the parasitized red blood cells maintained under the same conditions. The latter after 24 hours storage showed an O_2 uptake 88 per cent (72 to 100 per cent) of that of the fresh cells, while the former

TABLE II

Rate and Stability of Oxygen Uptake of Separated Parasites with Certain Substrates

Substrate	Concentration	Relative rate*		Per cent of initial rate remaining			
		No. of experiments	1st hr.	No. of experiments	2nd hr.	No. of experiments	3rd hr.†
	M						
None		25	10		1		
Glucose	0.006–0.012		100	33	89 (83–97)	24	81 (70–91)
Lactate	0.0125	7	109	5	81	3	63
Glucose	0.01			5	89	3	78
Pyruvate	0.002	8	95	3	86		
Glucose	0.01			3	92		
Succinate	0.008	11	31	10	62		
Glucose	0.01			10	90		
Fumarate	0.008	4	28	4	92		
Glucose	0.01			4	93		
Adenosine tri-phosphate‡	0.00024	4	29	3	80		
Glucose	0.01			3	89		
Adenylic acid‡	0.00024	7	26	4	80		
Glucose	0.01				93		

* Rates are here expressed relative to the rate of O_2 uptake with glucose as substrate, this being taken as 100.

† In several experiments with glucose as substrate the rate was observed for a longer period; per cent of initial rate 4th hour, 75 (62 to 80); 5th hour, 64 (55 to 75).

‡ See the text.

showed 76 per cent (71 to 82 per cent) of their original activity. In both cases, data only for cells stored with glucose were included in the average, since those stored in its absence had considerably less stability.

At 37°, the difference in stability was more marked. Parasitized red blood cells had a constant or even a slightly increasing O_2 uptake for at least 5 to 6 hours; the parasites, a rate linear for 60 to 90 minutes and thereafter slowly decreasing (Table II). Various substances, in addition to glucose and buffer, were added to the parasites in an attempt to increase their stability. These included yeast extract, duck plasma, duck red blood cell extract (prepared by dilution or by freezing and thawing),

succinate, 0.00024 M adenosine triphosphate (ATP) or adenylic acid, and a mixture of the known growth factors and vitamins. Some of these substances did increase the rate of oxygen uptake: yeast extract increased it about 20 per cent, succinate 30 per cent, fumarate 13 per cent, and plasma 10 per cent; the others had no effect on the rate. None led to an increased stability. Whether the slow decline in rate of O_2 uptake was due to damage to the parasites or to a defect in the medium remains undecided.

Substrates Utilized—With separated parasites, washed free from glucose after saponization, it was readily possible to demonstrate an oxygen uptake with certain other substrates (Table II), since with buffer alone such parasites showed only a small O_2 uptake that decreased nearly to zero in about 90 minutes. Only lactate and pyruvate gave a rate approximating that found with glucose but with none of the substrates was the rate as stable as with glucose; however, except in the case of succinate, the differences in stability were not marked. The results for each substrate are compared with the glucose results for the particular blood sample, inasmuch as differences in stability were found in parasites prepared from different samples of blood, while the results for any one blood sample were quite consistent. For convenience of representation, ATP and adenylic acid are included in the list of substrates, since their addition brought about an increase in O_2 uptake; in view of the fact that respiration in these instances had an R.Q. of 1.0, and was absent in controls to which had been added the products from ATP, initially heated at 100° for 10 minutes in N HCl, it seems probable that their's was the usual catalytic rôle, here associated possibly with the oxidation of some polysaccharide already present in the cells.[3]

Preparation of Pyruvate Solutions—In connection with the behavior of the parasites toward different substrates, it should be noted that at first erratic results were obtained with pyruvate. With buffer-washed parasitized red blood cells the use of pyruvate generally resulted in an oxygen consumption nearly as rapid as with glucose; on the other hand, with separated parasites there occasionally was obtained little or no O_2 uptake, and at other times the rate was approximately that observed with glucose as substrate. It was eventually noted that this variability lay in the pyruvate solutions rather than in the parasites. Those solutions that themselves afforded very low rates also inhibited more markedly the oxidation of glucose when added therewith. All solutions were prepared by dilution with ice water of pyruvic acid freshly distilled at 3 mm., followed by neutralization with sodium hydroxide to approximately pH 6.8. However, in some cases a slight excess of alkali was added before the desired endpoint was attained; it was found that these solutions gave the very low rates. Those prepared without even temporary overneutralization con-

[3] Analysis for reducing sugar (11) showed that no detectable glucose was present.

sistently showed an oxygen uptake 80 to 100 per cent of the glucose rate, when the final pyruvate concentration was 0.002 to 0.003 M. It was never possible to prepare a pyruvate solution that did not inhibit somewhat at higher concentrations.

In Table III are given the average results from several experiments, demonstrating the difference in behavior of exactly neutralized and transiently overneutralized pyruvate solutions as well as the differences in sensitivity thereto of separated parasites and parasitized red blood cells.

Respiration in Presence of Cyanide and Cresyl Blue—In an attempt to detect if possible the functioning of a flavoenzyme associated with respiration in the parasites, their behavior with cyanide and certain reversible

TABLE III

Oxygen Uptake of Parasites and Parasitized Red Blood Cells with Pyruvate As Substrate

Rates are given as percentages of the rate with glucose as sole added substrate.

Nature of pyruvate preparation	Pyruvate (0.002–0.003 M) and no glucose		Pyruvate (0.0125 M) + glucose (0.0115 M)	
	Parasites	Parasitized red cells	Parasites	Parasitized red cells
From freshly distilled pyruvic acid, not overneutralized	95 (80–105)		64 (40–67)	
From freshly distilled pyruvic acid, transiently overneutralized	14 (8–22)	94	34 (23–63)	93
From old pyruvic acid, not overneutralized	43 (23–63)	86	43 (40–46)	

dyes was studied. This segment of the investigation was motivated by the consideration that in tissues flavoprotein catalysis may in certain cases be "separated" from metalloenzyme catalysis by this procedure (12–15). In harmony with certain previous observations (8, 1) it was noted that the addition of 0.001 M cyanide nearly completely inhibited the oxygen consumption of the parasites, both as to separated material and parasitized red blood cells. Certain dyes, *e.g.* cresyl blue, thionine, methylene blue, toluidine blue, and toluylene blue, have been found partially to restore the oxygen consumption. Unfortunately these dyes also inhibit markedly the rate of oxidation in the absence of cyanide, so that they cannot be considered to be "ideal" substitutes for the iron catalysts. Cresyl blue appeared slightly less inhibitory and more catalytically active than the others (16) and was used in most of the experiments. It inhibited glucose oxidation about 35 per cent (25 to 45 per cent) and increased the rate in the

presence of cyanide from nearly zero (average, 7 per cent of the glucose rate) to 42 per cent (30 to 64 per cent) of the glucose rate.

The respiratory activity was less stable in the presence of cresyl blue, with or without cyanide, than in its absence, declining even during the 1st hour. The inhibitory effect of cresyl blue probably is most marked with respect to the later stages of glucose utilization, since the R.Q. in its presence is markedly reduced (Table IV). Furthermore, pyruvate oxidation in one experiment was inhibited 70 per cent by cresyl blue, and glucose oxidation only 45 per cent.

TABLE IV

Respiration of Parasites and Red Blood Cells in Presence of Cresyl Blue

Experiment No.	Type of cell	Control		HCN, 0.00125 M	Cresyl blue, 0.05 mg. per ml.		HCN, 0.00125 M; cresyl blue, 0.05 mg. per ml.	
		Rate	R.Q.	Rate	Rate	R.Q.	Rate	R.Q.
		microliters O₂ per 15 min.		*microliters O₂ per 15 min.*	*microliters O₂ per 15 min.*		*microliters O₂ per 15 min.*	
21	Separated parasites	27.1	0.83		14.8	0.53		
21	Parasitized red cells	33.2			42.5		11.9	
21	Normal red cells	6.3			14.7			
23	Separated parasites	13.9	0.71	1.3	9.4	0.57	8.9	0.57
24	" "	34.5	0.93	0.2			10.8	0.63
				NaN₃, 0.01 M			NaN₃, 0.01 M; cresyl blue, 0.05 mg. per ml.	
18	" "	12		4.3	9.8		8.6	

Parasitized red blood cells differ from the parasites in their behavior toward cresyl blue. Their rate of O₂ uptake is increased rather than decreased by the dye. This increase must be attributable in part to increased red cell respiration, since a similar effect is exhibited by normal red cells (Table IV; also (17, 18)). However, in the presence of cyanide and cresyl blue, the parasitized red cells resemble the parasites in that the resultant O₂ uptake is slow compared to the normal rate (Table IV).

In a few experiments sodium azide in place of cyanide was employed as respiratory metalloenzyme inhibitor (19). However, this reagent proved to be much less effective as inhibitor of O₂ uptake under the conditions, failing, even in a concentration of 0.01 M, to inhibit oxygen consumption

completely. With sodium azide, as with cyanide, the rate of oxygen uptake was partially restored by addition of cresyl blue. That the O_2 uptake with both cresyl blue and cyanide (or azide) approached the rate with cresyl blue alone, and further that the R.Q. was found to be very similar under both conditions, suggest that the restoration of respiratory activity to cyanide- or azide-treated cells is limited by an inhibitory effect of the catalytically active dyes upon one or more of the non-metalloenzymes involved in respiration.

Effect of Atabrine and Quinine—In agreement with the observations of other workers on parasitized red blood cells (3, 4), it was found that low

TABLE V

Effect of Atabrine and Quinine on Rate of Oxygen Uptake by Parasites

Type of cell	Substrate	No. of experiments	Atabrine			Quinine	
			0.0	0.001 M	0.0001 M	0.001 M	0.0001 M
				Inhibition			
				per cent	*per cent*	*per cent*	*per cent*
Separated parasites	Glucose, 0.011 M	5		51	24	50	4
	" HCN, 0.001 M + cresyl blue, 0.05 mg. per ml.	2		7	9	6	6
	Lactate, 0.0125 M	2			20		
	Pyruvate, 0.002–0.003 M	2		63	23		
	Succinate, 0.008 M	6			18		
	Fumarate, 0.008 "	5			15		
Parasitized red cells	Glucose, 0.011 "	3		55	22		
			respiratory quotient	*respiratory quotient*	*respiratory quotient*		
Separated parasites	" 0.011 "		0.93	0.88	0.88		

concentrations of atabrine and quinine had only a slight effect on the rate of parasite respiration, while higher concentrations, *e.g.* 0.001 M, did inhibit significantly. Since there was little change of R.Q. in the presence of atabrine, and since the inhibition did not differ markedly with various substrates, there was no immediate clue as to the stage of oxidation with which atabrine might interfere. In contrast to the action of cresyl blue, the characteristics of inhibition by atabrine are similar in parasitized red cells and in separated parasites.

In view of certain observations[4] (20, 21) on inhibitions of flavoenzyme

[4] Unpublished results of the authors.

activity, it seemed worth investigating whether interference by competition with the prosthetic group of one or more flavoproteins might account for the observed atabrine inhibition of parasite respiration. The study of "cyanide-cresyl blue respiration" permitted an opportunity for a test of such an assumption. It was found, however, that respiration under these conditions was practically unaffected in the presence of concentrations of atabrine and quinine as high as 0.001 M (Table V). It would seem, therefore, that if respiration in these cells under such conditions actually is mediated through a flavoprotein, the latter may be one that, like Straub's flavoprotein from heart muscle (15), is not subject to dissociation by atabrine.[4]

SUMMARY

1. A method is described for the separation of *Plasmodium lophurae* parasites from the red cells of parasitized duck blood. The separated parasites had an oxygen consumption that averaged 70 per cent that of the original blood. The rate of oxygen uptake with glucose as substrate was stable for 60 to 90 minutes at 37° and thereafter declined slowly.

2. Parasites washed free of glucose also showed an oxygen uptake with certain other substrates; with lactate and pyruvate the rate was approximately the same as with glucose; with succinate and fumarate it was 30 per cent of the glucose rate.

3. The oxygen uptake of the parasites was completely inhibited by cyanide and could be restored partially (40 per cent) by cresyl blue, although cresyl blue alone inhibited normal respiration to some extent.

4. The inhibition by atabrine and quinine of the oxygen uptake of the separated parasites with various substrates is described. Only at a concentration of 0.001 M was inhibition marked, approximately 50 per cent. There was no selective inhibition of the oxidation of glucose, lactate, pyruvate, succinate, or fumarate. The cyanide-cresyl blue respiration was not affected by atabrine and quinine.

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METABOLISM OF THE MALARIAL PARASITE, WITH REFERENCE PARTICULARLY TO THE ACTION OF ANTIMALARIAL AGENTS

II. ATABRINE (QUINACRINE) INHIBITION OF GLUCOSE OXIDATION IN PARASITES INITIALLY DEPLETED OF SUBSTRATE. REVERSAL BY ADENYLIC ACID*

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As described in Paper I (1), atabrine, except in relatively high concentrations, does not markedly inhibit the oxygen uptake of *Plasmodium lophurae* under the conditions there reported. However, it was subsequently noted that parasites that have been thoroughly exhausted of substrate oxidize glucose only after an induction period; in the presence of atabrine the oxygen uptake then is strongly inhibited at considerably lower concentrations than is the case with cells not deprived of glucose. A further study of this phenomenon was undertaken and is described below.

Methods

The erythrocyte-free *Plasmodium lophurae* parasites were obtained as previously described (1) from fresh, washed parasitized duck red blood cells, or from such cells after 24 hours storage at 0°, and were used within a few hours of the time of preparation. The only apparent difference between parasites prepared from fresh and from 24 hour red cells was a lower total activity in the latter case in which the rate of O_2 uptake with glucose averaged 82 per cent (70 to 94) that of parasites prepared from fresh red cells. In these experiments the cells were washed twice with buffer containing no glucose, four times the original cell suspension volume being used for each washing. The usual buffer (1) was used except for experiments that required phosphate analyses. Here, the parasites were washed with a salt solution of the following composition: KCl 0.0826 M, NaCl 0.0574 M, MgSO₄ 0.0015 M.

To complete the removal of oxidizable substrate from the parasites, they were suspended in glucose-free buffer, 0.6 ml. of cells, and 1.6 ml. of buffer in Warburg vessels and shaken at 37°, usually for 100 minutes; then

0.2 ml. of 0.07 or 0.14 M glucose was added from the side arm. Readings were taken at 15 minute intervals. Other additions were made initially or with the glucose, as indicated, the final volume being kept the same. For experiments in which changes in phosphate were followed, the usual phosphate-salt solution could not be used as buffer, and veronal proved to be strongly inhibitory. However, egg albumin, in a final concentration of about 2.5 per cent (3.4 mg. of N per ml.), was found to be an essentially inert and non-inhibitory buffer. It was prepared according to Kekwick and Cannan (2), dialyzed free from salt, and adjusted to pH 7.6 and to the desired protein concentration after determination of total nitrogen by Kjeldahl analysis. It was also adjusted to the same salt concentration as the above KCl-NaCl solution and then sterilized by Seitz filtration. In this way it could be kept in solution ready for use and free from bacterial contamination almost indefinitely at 0°.

Adenosine triphosphate (ATP) was prepared from rabbit muscle according to Needham (3). Adenylic acid (adenosine-5-phosphate) was prepared from ATP by $Ba(OH)_2$ hydrolysis, as described by Kerr (4). Crude coenzyme I (DPN) was prepared by the method of Williamson and Green (5) and was found to be 35 per cent pure. Flavin-adenine-dinucleotide (FAD) was prepared according to Warburg and Christian (6). A mixture of FAD and flavin mononucleotide was prepared by heating a solution of FAD for 10 minutes in N HCl at 100° (7). It was found to have decreased activity with d-amino acid oxidase and no benzyl alcohol-extractable pigment.

When glucose disappearance as well as O_2 uptake was to be followed, the contents of the Warburg vessels were transferred to centrifuge tubes, cooled, centrifuged, and the supernatants used for analysis for reducing ability by the Folin-Malmros method ((8), Umbreit (9) p. 103).

When changes in phosphorus (P) distribution were followed, a total volume of 3.2 ml., with proportionate increase in the parasite suspension volume from 0.6 to 0.8 ml., was used in each Warburg vessel. At the desired time the contents of three similarly treated vessels were combined and cooled. 1 volume of cold 16 per cent trichloroacetic acid was added, the mixture centrifuged and filtered, and 2 ml. of the filtrate used for each analysis. In a few cases parasites and supernatants were analyzed separately. The flask contents were centrifuged cold and the supernatants removed. The parasites were extracted with 8 per cent trichloroacetic acid and the supernatants precipitated with 1 volume of 16 per cent trichloroacetic acid. Aliquots of each fraction equivalent to 1 ml. of the original reaction mixture were used for the analysis. Since in most instances the absolute amounts, and in all cases the concentrations of all P fractions were considerably higher in the cells than in the supernatants,

the cells were not washed before extraction with trichloroacetic acid. Analyses for inorganic, total, and labile P were all carried out in duplicate, essentially as described by Le Page and Umbreit ((9) p. 163).

Results

In Table I are summarized the results of numerous experiments on the rate of oxygen uptake after addition of various substrates to parasites that initially were shaken for 100 minutes in substrate-free buffer. In order to average the results of experiments done at different times, all the

TABLE I

Rate of Oxidation of Various Substrates by Parasites Initially Depleted of Substrate

Substrate	Relative rates*					Per cent inhibition of final rate by 0.0001 M atabrine	
	Substrate added to non-depleted cells	Substrate added after depletion					
		No. of experiments	0–15 min.	15–30 min.	Final		
	M						
Glucose	0.006	100	23	8	34	59	80 (50–95)
" †	0.006	100	11	6	26	57	87 (83–95)
Lactate	0.0125	109	4	41	59	61	20 (15–25)
Pyruvate	0.002	95	4	20	54	58	38 (10–60)
Succinate	0.008	31	2	17	25	15	38 (36–40)
Fumarate	0.008	28	2	9	21	15	0 (+20 to −20)
Adenosine triphosphate‡	0.00024	29	2	1.5	16	13	15 (0–30)
Adenylic acid‡	0.00024	26	3	9	17	14	16 (16–17)

* Rates relative to the rate of oxidation of glucose by the same parasite preparation, taken as 100, when the glucose was added simultaneously with the parasites.

† These comprise tests solely with those parasite preparations that were studied with the other substrates. The first line includes all glucose tests (see the text).

‡ Included here for convenience of representation; compare foot-note 1.

rates were calculated relative to the rate of oxidation of glucose by the same parasite preparation, when the glucose was added to the reaction mixture simultaneously with the parasites. Moreover, in averaging the results for this and Tables II to X, duplicate experiments with parasites from any one blood sample, even if conducted on different days, have been counted as one experiment, since the results always agreed closely except for a gradual decrease in absolute activity with age. On the other hand, especially with regard to their sensitivity to atabrine, different blood samples varied. The column headed "Final" in Table I refers to the rate 40 to 90 minutes after addition of substrate to depleted cells. By this time the cells to which glucose had been added had achieved their maximum, relatively constant rate of O_2 uptake.

With all substrates the rate during the first 15 minutes was less than the final rate.   However, the difference was conspicuously greater for glucose than for the other compounds, with the exception of ATP,[1] with which the delayed increase in O_2 uptake probably was associated with the impermeability of the cells to the ATP itself.[2]   Pyruvate also gave evidence of a slight incubation period during this interval.   On the basis of the more reliable data for the second 15 minute period it is clear that only in the case of glucose is there a pronounced induction period.   The final rate with each substrate was only 50 to 60 per cent of that obtained when the substrate was added initially, without depletion, indicating that in addition to the reversible inhibition chiefly of glucose oxidation, some irreversible damage affecting the behavior of the parasites toward all substrates had taken place during the substrate depletion period.   The duration of the induction period on addition of glucose and the extent of final recovery varied with the length of the initial substrate-free period.   The shorter this period, the shorter the subsequent induction period and the greater the extent of recovery.

The induction period could be shortened considerably if there was added during the depletion stage, or together with the glucose after depletion, any one of the following: fumarate, succinate, adenylic acid, or ATP (Table II).   The four substances were approximately equally effective if present *during the depletion period*.   In the case of addition simultaneously with glucose, the rate was at once increased by succinate, fumarate, or adenylic acid, but less rapidly by ATP, an effect again occasioned probably by the impermeability of the cells to ATP.   The increased rate of O_2 uptake during the first half hour was greater than could be accounted for by a mere summation of the two rates; *i.e.*, for glucose and the second substance.   The final rate with succinate or fumarate was significantly greater than the control, and only slightly greater with adenylic acid or ATP, as might have been anticipated from the fact that the first two have been observed generally to exert an additive action when used with glucose, while the latter two have not (1).

Atabrine Inhibition after Substrate Exhaustion—When atabrine (initial concentration 0.0001 M) was present during the depletion period, or even if it was added with the substrate, the recovery of oxygen uptake with glucose usually was inhibited 80 to 90 per cent.   This was in marked contrast to the 20 per cent inhibition observed with this concentration of atabrine when glucose was used with non-depleted parasites (1).   The average inhibition with substrate-exhausted parasites from twenty-five

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[1] Adenylic acid, added as such or furnished through hydrolysis of added ATP, possibly accelerated oxidation of an intracellular substrate (1).

[2] This is discussed later.

blood samples was 80 per cent. With the majority, twenty-one, the inhibition varied between 78 and 95 per cent, average 85 per cent; the other four showed an average inhibition of only 55 per cent (50 to 70 per cent). A few experiments (Table III) were performed in which the total atabrine concentration was varied. These indicated that there was a rather abrupt

TABLE II

Effect of Succinate, Fumarate, Adenylic Acid, and Adenosine Triphosphate (ATP) on Duration of Incubation Period

All rates are given as per cent of the rate with glucose when this is added before depletion.

Substance added	Present during depletion					Added with glucose					
	Concentration	No. of experiments	Relative rate			No. of experiments	Relative rate after addition of glucose				
			During depletion period	After addition of glucose			0–15 min.		15–30 min.		
				0–15 min.	Final		Observed	Calculated*	Observed	Calculated*	Final
	M										
Succinate	0	9	14	8	64	4	9		43		66
	0.008		32	56	88		41	26	87	68	84
Fumarate	0	4	11	7	62	5	11		37		62
	0.008		28	56	86		17	20	59	38	74
ATP	0	4	11	13	71	7	3		27		55
	0.00024		29	65	79		7	5	57	43	55
" acid-treated†	0	1	11	8	61						
	0.00024		13	13	59						
Adenylic acid	0	7	11	5	57	6	4		28		53
	0.00024		26	51	69		25	13	63	45	59
Yeast adenylic acid	0	1	9	7	65	2	4		38		55
	0.00024		11	15	73		15		39		57

* From the sum of the control value and the value given for the particular substrate in Table I.

† Heated 10 minutes at 100° in N HCl.

change in inhibition with increase in concentration, the concentration at which this change occurred varying with different preparations.

The cause of this difference in sensitivity in parasites from certain blood samples is not known. It may be that the inhibition depends not upon the total atabrine added, but rather on the actual concentration in the parasites, and that this in turn varies with the free atabrine concentration. The latter quantity was estimated by analysis (10) of the supernatant after removal of the parasite-nuclei mixture by centrifugation. With a

total atabrine concentration of 1×10^{-4} M, there was found in one experiment a free atabrine concentration of 0.05×10^{-4} M and in a second 0.1×10^{-4} M. A total atabrine concentration of 3×10^{-4} M gave a free atabrine concentration of 0.4×10^{-4} M in both instances. With nuclei from normal blood, prepared in the same way as the parasite-nuclei mixture, 10^{-4} M atabrine added gave a free concentration of 0.16×10^{-4} M. These results indicate that the free atabrine was decidedly lower than the total. A large portion of the atabrine is removable by the nuclei, and may be unavailable for the parasites. It is conceivable that there might be variation in the amount of atabrine bound by the nuclei, and, since this is so large a proportion of the total, small variations might lead to greater variations in free atabrine and hence in the effectiveness resulting from any particular initial concentration. Whether there would be better

TABLE III

Relation between Concentration of Atabrine and Adenylic Acid and Inhibition of Glucose Oxidation in Substrate-Depleted Parasites

Atabrine						
0.00003 M	0.0001 M			0.0003 M		
		Adenylic acid				
		0.00012 M	0.00024 M		0.00012 M	0.00024 M
			Per cent inhibition			
82	89	31	9			
10	79	7		79	29	
22	81	78	23	85		
62	86		32	88		43

quantitative correlation between the degree of inhibition and the *free* atabrine concentration is not known.

It can be seen from the data in the last column of Table I that the atabrine inhibition of oxygen uptake by substrate-depleted cells is very much more marked with glucose than with any other substrate. When, in place of glucose, there was added after depletion lactate or fumarate, the inhibition by atabrine was no greater than when these substrates were used with non-depleted cells (1). With succinate there was a somewhat greater inhibition, but the absolute rates with this substrate were almost unreliably low under the new conditions. Pyruvate oxidation by the depleted cells also showed an increased sensitivity to atabrine. However, the magnitude of this sensitivity has not been thoroughly explored. The results were more variable than with glucose, and in no case was the final inhibition as great.

It must be emphasized that the atabrine "block" was observed only with separated parasites. When parasitized red blood cells were washed several times with glucose-free buffer and resuspended in buffer, their oxygen uptake was reduced to about 25 per cent of the rate with glucose and this rate declined only slowly during the course of 3 to 4 hours, in contrast to the rapid decline of the rate with washed, substrate-free parasites to nearly zero in about 100 minutes. Since it was impracticable here to obtain

TABLE IV

Antagonism by Various Substances; Glucose Oxidation in Substrate-Depleted Parasites

	Time of addition	No. of experiments	Per cent inhibition by 0 0001 M atabrine	
			Control	Plus antagonist (1st column)
	min.			
ATP,* 0.00024 M	0	4	88 (78–93)	32 (25–39)
	100	5	88 (81–92)	34 (25–39)
	100	1	88	72
Adenylic acid, 0.00024 M	0	7	85 (79–92)	25 (7–33)
	0	1	84	77
	100	9	84 (71–93)	25 (3–45)
Succinate, 0.008 M	0	3	83 (79–90)	45 (40–52)
	0	3	88 (83–95)	80 (77–85)
	100	2	84 (80, 87)	74 (67, 82)
Fumarate, 0.008 M	0	2	84 (80, 88)	38 (35, 40)
	0	3	80 (76–83)	68 (66–71)
	100	3	86 (83–89)	72 (70–76)
Yeast adenylic acid, 0.00024 M	0	1	88	89
	100	2	72 (56, 88)	70 (54, 86)
Crude DPN,† 160 γ per ml.	0	1	79	35
Same, autoclaved	0	1		40
ATP, 0.00024 M	0	1	90	35
Same, acid-treated‡	0	1		88

* Adenosine triphosphate.
† See the text.
‡ Heated 10 minutes, N HCl, 100°.

substrate-depleted parasitized red cells, glucose was added after a 3½ hour period, when the rate was still 15 per cent of the control, whereupon the O_2 uptake at once increased and reached its maximum value with only a slight induction period. Under the conditions, the recovery was hardly significantly more sensitive to atabrine than was the normal oxygen uptake.

Reversal of Atabrine "Block" by Adenylic Acid—The effect of atabrine upon glucose utilization in parasitic cells initially substrate-depleted can be partially or completely prevented by the addition of adenylic acid or ATP (Table IV). There is some variation in the extent of the reversal,

but in only two of the eighteen blood samples tested was the inhibition not significantly decreased by the one or the other. Variation in response to any fixed concentrations of inhibitor and antagonist would be anticipated from the variations in atabrine sensitivity and the dependence of the degree of reversibility upon both atabrine and adenylic acid concentration

TABLE V

Comparison of Effect of Adenylic Acid and Fumarate on Atabrine Inhibition in Substrate-Depleted Parasites

The rates, except for the values in parentheses, are those attained 60 to 120 minutes after addition of the indicated substances to substrate-depleted cells, at which time those to which glucose alone had been added had reached their maximum rate of O_2 uptake. In the last two experiments fumarate and adenylic acid, when present, were added at the start of the depletion period and the values in parentheses are the rates of oxygen uptake during that period, before addition of glucose. All rates are given in terms of microliters of O_2 per 15 minutes.

Experiment No.	Initial rate with glucose*		Atabrine									
			Fumarate added from side arm		Adenylic acid added from side arm		Glucose added from side arm					
							No other addition		With fumarate		With adenylic acid	
			0	0.0001 M	0	0.0001 M	0	0.0001 M	0	0.0001 M	0	0.0001 M
	microliters O₂ per 15 min.											
50	30.4	Final rate	5.0	3.8	4.3	3.6	17.8	3.0	23.0	6.6	18.0	13.2
		% inhibition		22		16		83		71		26
42	28.3	Final rate	5.5				17.4	2.6	19.0	5.8	18.7	11.3
		% inhibition						85		70		40
			Fumarate added with parasites		Adenylic acid added with parasites							
38	28.3	Final rate	(8.9)	(7.7)	(6.5)	(5.7)	19.6	2.3	26.6	16.1	22.7	14.4
		% inhibition		(14)		(12)		88		40		36
60	24.8	Final rate	(5.2)	(4.5)	(6.0)	(5.8)	12.4	2.1	19.6	6.6	13.6	9.2
		% inhibition		(12)		(3)		83		66		32

* Glucose added before substrate depletion.

(Table III). The averaged results given in Table IV indicate also a fairly frequent reversal by succinate and fumarate. When comparisons were made between adenylic acid and succinate or fumarate with the same parasites, the atabrine antagonism usually was decidedly more marked with the former (Table V), although in a few experiments fumarate appeared to bring about as good reversal of the inhibition as did adenylic acid (Table V, Experiment 38). From these results it might be supposed that

any substance capable of producing an increased oxygen consumption could in a non-specific way lead to decreased cell damage and consequently decreased atabrine sensitivity. However, this probably is not the chief explanation. The most efficient agents, adenylic acid and ATP, do not appear to prevent cell damage, as reflected by the extent of recovery of the rate of oxygen uptake (Table II). Also they are effective whether added before or after substrate depletion, whereas protection by succinate and fumarate is observed only when these substances are present throughout the experiment. The decrease in atabrine sensitivity under the latter conditions may be related to frequent observations (11–13) that the oxidation of numerous substrates is associated with phosphorylation and would tend to maintain a high concentration of ATP.[3]

In view of the rather unexpected increase in O_2 uptake observed upon the addition of adenylic acid or ATP *alone* to substrate-free parasites (1), it seemed necessary to find some evidence that the effects of these compounds were not to be attributed to some impurity. Therefore four different preparations of adenylic acid were tested simultaneously. Two were prepared in this laboratory from two different ATP preparations, one of ·these being recrystallized once and the other six times. The other samples of muscle adenylic acid were obtained from Dr. Mary V. Buell. They had been isolated from heart muscle at different times (14). Identical results with respect to (a) rate of oxygen uptake in the absence of substrate, (b) shortening of the incubation period, and (c) antagonism of atabrine action were obtained with all preparations upon three different parasite preparations from two different blood samples. Furthermore all of these effects of adenylic acid were abolished by 10 minutes hydrolysis of this nucleotide with N HCl at 100°, a treatment known to destroy the adenine-pentoside linkage (Tables II and IV).

Various other substances were tested for their effect upon glucose oxidation. The following had no influence at all upon the rate during the depletion period, the length of the induction period, or the inhibition by atabrine: adenine 0.0013 M, spermine or spermidine 0.0004 M, pyridoxine 0.17 mg. per ml., a mixture of flavin mono- and dinucleotides 1.7 γ per ml., cocarboxylase, and a mixture containing adenine, guanine, uracil, xanthine, thiamine, nicotinamide, nicotinic acid, p-aminobenzoic acid, pyridoxine, pantothenate, riboflavin, choline, and ribose. Yeast adenylic acid (adenosine-3-phosphate) also was without effect. The crude DPN preparation proved capable of antagonizing atabrine; however, this effect was unaltered by autoclaving at 120°, and thus could not have been attributable to DPN itself.

[3] See "Discussion."

Action of Various Agents; Adenylic Acid As Antagonist (Table VI)—A few other agents were tested for their effect on the recovery of substrate-depleted cells and several, like atabrine, markedly inhibited this process in a concentration that was only slightly or not at all inhibitory with cells

TABLE VI

Inhibition of Recovery of Oxygen Uptake in Substrate-Depleted Cells by Various Agents

Inhibitor	Glucose added before depletion		Glucose added after substrate depletion					
			Inhibitor (1st column)			Atabrine		
	Inhibitor	Atabrine	Concentration		Plus adenylic acid	Concentration		Plus adenylic acid
	Inhibition			Inhibition			Inhibition	
	per cent	*per cent*	M	*per cent*	*per cent*	M	*per cent*	*per cent*
Quinine	15	25	0.0003	86	46	0.0001	88	34
Plasmochin	−13		0.0003	82	28	0.0003	82	77
						0.0001	71	37
"			0.0003	90	34			
		4	0.0001	90		0.0001	88	33
			0.00003	88		0.00003	82	
SN6911*			0.0001	81	0	0.0001	85	15
SN10,447†			0.0001	90	0	0.0001	82	
Methoxyquinoline	−12		0.001	0				
	−20	16	0.0003	8	−14	0.0001	85	35
Sulfanilamide	0		0.01	8		0.0001	86	8
Sulfathiazole			0.01	54		0.0001	88	9
			0.005	22	8	0.0001	85	15
Auramine			0.0002	79	45	0.0001	49	
SN12,710-6029‡	−10		0.0002	51	6	0.0001	49	
SN11,527§			0.001	5	0	0.0001	71	37

* The Survey number, designated SN, identifies a drug in the records of the Survey of Antimalarial Drugs. The antimalarial activities of those compounds to which Survey numbers have been assigned will be tabulated in a forthcoming monograph. SN6911 refers to 3-methyl-4-(4-diethylamino-1-methylbutylamino)-7-chloro*quinoline* diphosphate.

† 4-(4-Diethylamino-1-methylbutylamino)-2,3-dimethylquinoline diphosphate.

‡ "Novalauramine" (Hellerman, L., and Porter, C. C., to be published).

§ N-(4-Diethylamino-1-methylbutyl)-β-(p-dimethylaminophenyl)*alanine* (Hellerman, L., and Porter, C. C., to be published).

never deprived of glucose. These included quinine, plasmochin, auramine, "novalauramine," and several others.[4] Sulfathiazole showed a slight inhibition only in very high concentrations (0.01 M). Sulfanilamide, 6-methoxyquinoline, and the complex amino acid, SN11,527, had no effect (Table VI). In view of the variability noted in atabrine sensitivity, the

[4] Structures given in Table VI.

effect of each compound was compared with that of atabrine upon the same parasites.

Oxygen Uptake in Relation to Glucose Disappearance—As a check on the significance of the oxygen uptake measurements, glucose disappearance was measured simultaneously in a few experiments. The results of the glucose analyses were in agreement with the oxygen uptake measurements (Table VII).

Atabrine and Adenylic Acid with Non-Depleted Cells—In contrast to the effect of adenylic acid or ATP on substrate-depleted cells is their com-

TABLE VII

Glucose Utilization in Substrate-Depleted Cells

Experiment No.	Antagonist	Initial glucose	Glucose consumed				Maximum rate of O₂ consumption per 15 min.		
				Plus atabrine, 0.0001 M				Plus atabrine, 0.0001 M	
						Inhibition			Inhibition
		γ	γ	γ	γ	per cent	microliters	microliters	per cent
54*		210	166	22	87		19.1	2.5	87
	Adenylic acid, 0.00024 M	210	168	168	0		19.6	16.6	15
60†		404	248	33	87		10.8	2.6	76
	Fumarate, 0.008 M	404	338	39	88		17.5	5.5	69
	Adenylic acid, 0.00024 M	404	358	195	45		14.0	8.2	42

* Adenylic acid and atabrine were both added from the side arm with the glucose 100 minutes after placing the parasites in the bath. The maximum rates were the rates 30 to 60 minutes after mixing, since thereafter they declined, probably due to lack of substrate. The flask contents were removed for analysis 100 minutes after mixing.

† Adenylic acid, fumarate, and atabrine were added to the parasites immediately before they were placed in the 37° water bath. 100 minutes later the glucose was added. The maximum rate was the rate 45 to 100 minutes later. The flask contents were removed for analysis 100 minutes after the addition of glucose.

plete lack of effect on the atabrine inhibition of the oxygen uptake of cells never deprived of glucose (Table VIII).

Phosphate Distribution—In view of the apparently specific action of adenylic acid in antagonizing the action of atabrine and certain other compounds in substrate-depleted cells, it seemed of interest to attempt to follow alterations in the constituent phosphate compounds under various conditions. Owing to the low total phosphate concentration of the reaction mixtures and to the limited amount of parasitized blood available, fractionation and identification of the phosphate compounds present were

not possible.   Only changes in inorganic, labile, and total phosphate were followed and, since the latter two involve the difference between two analyses, the results are probably not accurate to more than $\pm 0.5 \, \gamma$ of P.   The changes observed in the P fractions under different conditions were, however, qualitatively quite consistent and are therefore significant, especially since nuclei from normal red blood cells contained no acid-soluble organic P and only traces of inorganic P under any conditions.

The results of a few typical experiments are given in Table IX.   Experiments 45 and 48 illustrate the changes in P distribution observed when trichloroacetic acid filtrates of the entire reaction mixtures were analyzed. As is seen in Experiment 45, C and G, there was little significant change in P distribution during 100 minutes incubation at 37° *with glucose*, except for a slight increase in inorganic and total acid-soluble P.   However, when the

TABLE VIII

Effect of Adenylic Acid or Adenosine Triphosphate (ATP) on Atabrine Inhibition of Oxygen Uptake in Glucose-Saturated Parasites

	Rate of O_2 uptake per 15 min.			
Experiment No.	No atabrine		Atabrine	
	Glucose	Glucose plus adenylic acid or ATP	Glucose	Glucose plus adenylic acid or ATP
	microliters	*microliters*	*microliters*	*microliters*
47*	32.9	32.1	13.6	13.7
50†	30.4	30.8	19.9	20.7

* Atabrine, 0.0005 M; ATP, 0.00024 M.
† Atabrine, 0.0003 M; adenylic acid, 0.00024 M.

cells were incubated without glucose, there was, as might have been anticipated, a large increase in inorganic P and a decrease in both organic and labile P.   There was also an increase in total acid-soluble phosphate, owing possibly in part to increased cellular disintegration under these unfavorable conditions.   When glucose was added to such substrate-depleted cells, and the mixture incubated for 90 minutes more, there was no further increase in total acid-soluble P, a small increase in organic, a decrease in inorganic, and an increase in labile P to close to the value found with cells incubated with glucose without initial depletion.   Addition of atabrine to cells during the first 100 minutes incubation either with or without glucose caused no significant changes in P distribution, as compared to the corresponding controls, except for the increase in labile P found in the glucose-atabrine-treated cells.

In Experiments 56 and 58 cells and supernatants were analyzed separately.   The results for both factions are given as micrograms per ml. of

the original reaction mixtures, since the small cell volume was not accurately measured. The figures are therefore proportional to the relative amounts rather than to the concentrations in cells and supernatant. For the cells, the changes in organic, inorganic, and labile P under different conditions were similar to those already noted in analyses of whole mixtures.

TABLE IX

Phosphate Fractions in Parasite Reaction Mixtures

Experiment No.	Type of treatment*	Phosphate per ml. reaction mixture							
		Total		Inorganic		Organic		Labile	
		γ		γ		γ		γ	
45	C	7.3		2.1		5.2			
	G	8.4		3.3		5.1			
	B	10.8		7.8		3.0			
48	G	12.4		4.0		8.4		3.4	
	AG	13.4		4.6		8.8		4.1	
	B	16.5		10.8		5.7		1.5	
	AB	17.5		11.5		6.0		1.6	
	BG	15.8		8.6		7.2		3.0	
		Cells	Supernatant	Cells	Supernatant	Cells	Supernatant	Cells	Supernatant
56	G	8.5	3.3	1.7	2.4	6.8	0.9	2.0	0
	B	11.6	6.7	5.6	4.0	6.0	2.7	1.5	0
	BG	10.6	8.5	3.3	5.9	7.3	2.6	2.3	0
	ABG-1	8.9	10.1	5.7	7.1	3.2	3.0	0.5	0
58	G	8.0	5.1	1.5	2.8	6.5	2.3	2.4	0
	B	9.2	6.8	5.6	4.6	3.6	2.2	1.0	0
	BG	8.7	8.1	2.9	5.1	5.8	3.0	2.7	0
	ABG-2	9.3	7.9	4.3	5.9	5.0	2.0	2.1	0.1

* C, freshly prepared parasites diluted with egg albumin-chloride buffer as for O_2 uptake measurements. In other experiments the parasites were shaken in Warburg vessels at 37° with additions and times as follows: B, no substrate, 100 minutes; G, glucose 0.006 M, 100 minutes; BG, no substrate, 100 minutes, then glucose, 90 minutes; AG, glucose 0.006 M plus atabrine 0.0005 M, 100 minutes; AB, atabrine 0.0001 M, no substrate, 100 minutes; ABG-1, no substrate, 100 minutes, then glucose 0.006 M plus atabrine 0.0001 M, 90 minutes, 80 per cent inhibition of final rate; ABG-2 no substrate, 100 minutes, then glucose 0.006 M plus atabrine 0.000075 M, 90 minutes, 45 per cent inhibition of final rate.

The changes in the supernatant fractions were less characteristic. In general the total extracellular P increased as the total incubation time was prolonged, and under all conditions the inorganic P comprised 60 to 70 per cent of the total extracellular P. However, it is noteworthy that although some organic P was always found in the supernatant, labile P was never present.

As mentioned above, identification of the P compounds in the parasites under different conditions was not possible.   However, attempts were made to identify some of the P fractions in parasites freshly prepared from 30 to 40 ml. of parasitized blood.   The centrifuged parasites were extracted once with 10 per cent trichloroacetic acid and then the barium salts were precipitated from the extract at pH 8.2 by addition of barium acetate and alcohol.   The precipitate was dissolved in dilute acid and the water-insoluble salts precipitated at pH 8.2.   Both the soluble and in-

TABLE X

Analysis of Water-Insoluble Barium Salts in Trichloroacetic Acid Extract of Parasites

		Experiment I	Experiment II
Total volume of blood sample, *ml*		44	30
Parasite count, $\times 10^8$ per *ml.*		12.5	11.5
Rates of O_2 uptake with glucose, *microliters*			
O_2 per 15 min. per 0.6 ml. parasites		34.7	29.1
Organic P, *micromoles*		11.6	6.98
Labile " "		8.7	5.59
Organic " "		14.9	9.11
Pentose, *micromoles*		4.15	3.19
Nitrogen, "		18.9	15.3
Reducing sugar			0
	Calculated for adenosine triphosphate		
$\dfrac{\text{Labile P}}{\text{Organic P}}$	0.67	0.58	0.61
$\dfrac{\text{Labile P}}{\text{Pentose}}$	2.0	2.1	1.8
$\dfrac{\text{N}}{\text{Pentose}}$	5.0	4.5	4.8
Labile P in original extract, *micromoles*		17.4	11.8
Recovery in this fraction, %		50	48

soluble fractions were nearly completely consumed by analyses for inorganic, labile, and total P, and pentose (15), fructose (16), nitrogen, and free reducing sugar (Umbreit (9) p. 103).   Therefore no further purification of either fraction was possible.   No conclusions could be drawn from the analyses of the water-soluble barium salts, but analysis of the insoluble fraction suggested that it consisted largely of inorganic P and adenosine triphosphate.   The latter represented about 50 per cent of the labile P present in the original acid extract (Table X).

DISCUSSION

A tentative explanation of the results may be offered if it is assumed that the carbohydrate metabolism of *Plasmodium lophurae* is similar to that of

certain other cells; *e.g.*, mammalian tissue cells. This assumption receives general support from the work of Speck and Evans (17), who showed that various enzymes with function similar to those involved in the carbohydrate metabolism of tissue cells can be extracted from *Plasmodium gallinaceum* parasites. Glucose is the only substrate whose utilization appears markedly to be delayed after substrate depletion. Since, moreover, its oxidation can be initiated more rapidly if ATP or adenylic acid is added, it seems probable that the induction period is to be attributed to the requirement for phosphorylation of glucose before the latter can be utilized. This process occurs rapidly only with adequate ATP or when some coupled oxidation is taking place. In the fermentation of glucose by yeast extracts, it is known that a similar induction period can be decreased by small amounts of hexose diphosphate (18) which evidently initiates the oxidation-phosphorylation cycle. We have found 1,6-diphosphofructose to be without effect on the parasites, owing probably to their impermeability to this substance. Cellular impermeability to ATP may account also for the greater effectiveness of adenylic acid in shortening the induction period under certain conditions (Table II). The phosphate analysis of cells and supernatants indicates that no labile phosphate can escape from the cells, although there was escape of some stable organic P; presumably added ATP must have undergone preliminary hydrolysis in the reaction mixtures. The shortening of the induction period by succinate and fumarate may be assignable to an increased rate of formation of ATP from adenylic acid in the cells; in mammalian tissue their oxidation is accompanied by phosphorylation (11–13).

If the above explanation of the induction period is correct, atabrine must interfere with some phosphorylation reaction. The antagonism to the effect of atabrine exhibited by adenylic acid and ATP suggests that atabrine may compete with one or both for some enzyme. In cells already metabolizing glucose the relatively lower degree of inhibition by atabrine is not affected by an increased adenylic acid concentration. In such cells the adenylic acid presumably is kept chiefly in the form of ATP during the continuous oxidation of glucose. Under these conditions adenylic acid would not escape, and a high concentration of ATP would be maintained; thus any competitive effect of atabrine would be negligible. In parasitized red blood cells incubated for 3 to 4 hours without substrate the absence of an induction period and of significant inhibition by atabrine of the oxidation of glucose may also be attributable to conservation of ATP accompanying their low but continuous rate of oxygen utilization. A similar effect may account for the occasional prevention by fumarate of atabrine inhibition in separated parasites deprived of glucose.

Interference by atabrine with a phosphorylation would be not incon-

sistent with the results of Speck and Evans (17) relative to the sensitivity to atabrine of various enzymes in *Plasmodium gallinaceum.* These authors found that hexokinase, which catalyzes the phosphorylation of glucose by ATP, was more strongly inhibited by atabrine than were any of the other identifiable enzymes of carbohydrate metabolism. There was no evidence as to the effect of excess ATP in relation to the inhibition.

Whether the kind of inhibition by atabrine described in this paper is at all associated with its *in vivo* activity is not certain. Against such a hypothesis is the observation that the inhibition has been observed to occur only in erythrocyte-free parasites deprived of glucose. The phenomenon may not be related to the inhibition of "normal" respiration; *i.e.,* in nondepleted cells, where adenylic acid has appeared to be devoid of any effect (Table VIII).

On the other hand it is possible that even in the presence of glucose atabrine may cause interference with some phosphorylation reaction essential to the life or reproduction of the cell. Such a phosphorylation would not necessarily be rate-limiting with respect to respiration. The inhibition of normal respiration by atabrine might then be attributable to some other reaction. The assumption, admittedly hypothetical, that the mode of action of an agent on any one organism may not be always the same when conditions are varied, is in accord with observations of the sensitivity of a wide variety of enzymes to the action of atabrine and other antimalarial drugs (17, 19–21).[5]

SUMMARY

1. When glucose is added to erythrocyte-free *Plasmodium lophurae* parasites that initially have been washed free from glucose and incubated 100 minutes at 37° in the absence of substrate, oxygen uptake is resumed only slowly. The induction period is decidedly less marked with the other substrates, lactate, pyruvate, succinate, and fumarate. The recovery of oxygen uptake by such cells on addition of glucose is inhibited usually 75 to 90 per cent by atabrine initially 0.0001 M, a concentration that causes only a 20 per cent reduction in rate of oxygen uptake by cells that have not been first deprived of glucose. This marked inhibition is not observed with substates other than glucose. An effect similar to that of atabrine is observed with quinine, plasmochin, and other related drugs; not with sulfanilamide, sulfathiazole, or 6-methoxyquinoline.

2. The atabrine inhibition of oxygen uptake by substrate-depleted cells can be prevented partly or completely by adenylic acid or adenosine triphosphate.

[5] Unpublished results of the authors.

3. Determination of glucose utilization by analysis for reducing sugar confirms the above results obtained by measurements of the rate of oxygen uptake.

4. Analysis for changes in total, labile, and inorganic phosphorus of parasite reaction mixtures under various conditions indicates that 100 minutes incubation in buffer, without substrate, leads to an increase in inorganic and total acid-soluble phosphorus and to decrease in organic and labile phosphorus. On subsequent addition of glucose the changes in organic, inorganic, and labile P are partially reversed, unless atabrine is present in concentration sufficient to prevent oxygen uptake. Separate analysis of cells and supernatant indicates that, although some organic phosphorus appears in the supernatant, no labile phosphorus is ever present outside the cell.

5. It is concluded that the induction period in the oxidation of glucose by substrate-depleted cells is attributable to the necessity for phosphorylation of glucose before this substrate can be utilized, and that atabrine interferes with this phosphorylation, possibly by competition with adenylic acid, adenosine triphosphate, or both.

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FLAVOENZYME CATALYSIS

INHIBITION OF d-AMINO ACID OXIDASE BY COMPETITION WITH FLAVIN-ADENINE-DINUCLEOTIDE OF ATABRINE (QUINACRINE), QUININE, AND CERTAIN OTHER COMPOUNDS*

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Previous studies in this laboratory on the catalysis by the flavoenzyme, d-amino acid oxidase, have been concerned with the specificity of the enzyme[1] and with an inhibition of its activity by a group of compounds[2] the members of which act apparently to affect the orientation of the substrate to the complete enzyme. In the latter investigation it was found that the characteristics of the competition displayed by the benzoate ion (1) with respect to the substrate are exhibited in some degree by a large number of compounds incorporating the conjugate system[3]
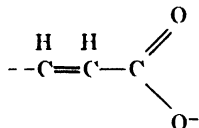
The phenomena relating to inhibitions of biocatalytic systems, and particularly the reversible actions thereupon, are of manifest current interest with respect to the mode of action of certain compounds. In addition, there recently has been considerable interest in the antagonism to the action of some chemotherapeutic agents that is exhibited by certain growth factors of similar structure. It seemed possible that the action of certain antimalarial drugs upon malarial parasites might also be related to some such competitive effect. To this problem an approach may be made with respect

[1] Hellerman, L., Lindsay, A., Weisiger, J. R., and Ramsdell, P., unpublished data.

[2] Hellerman, L., and Lindsay, A., unpublished data.

[3] It would appear that the presence of this configuration as an element in the structure of the inhibitor predisposes the latter for reversible combination with characteristic acceptor group or groupings of the complete enzyme, in competition with the substrate (see foot-note 2).

to the potential interference of various substances in the metabolic processes of living or "surviving" cells (2, 3) as well as to reversible or irreversible actions upon isolated enzymes. Our work with parasitic cells has been paralleled with studies of various enzymes including catalase, lipase, and several flavoenzymes, especially d-amino acid oxidase, an available, relatively stable representative of the flavoproteins capable of dissociation. Meanwhile, the observations of Wright and Sabine on the lowering of an atabrine inhibition of tissue respiration and of d-amino acid oxidase activity by flavin-adenine-dinucleotide (4) and the similar finding of Haas (5) with respect to cytochrome reductase of yeast conveyed suggestions that atabrine might be capable of competition *in vivo* for one or more essential proteins with flavin nucleotides, which are combined forms of the vitamin riboflavin. In this paper is described an investigation of d-amino acid oxidase in a quantitative study of the effect of atabrine and quinine and a more or less qualitative comparison of the action of related compounds.

Our results do not uphold a hypothesis of a specific antagonism between atabrine and FAD, comparable to the sulfonamide-p-aminobenzoate relationship (6, 7). They indicate rather that atabrine, quinine, and the sulfonamides all belong to a class of compounds capable of combination with proteins. This combination may, with certain enzymes, result in competition for the prosthetic group, but such competition cannot be attributed to a close structural similarity between inhibitor and prosthetic group. Moreover the extent of inhibition by these compounds cannot always be interpreted on the assumption of a completely reversible combination.

Methods

d-Amino acid oxidase was prepared from lamb kidneys and separated from the prosthetic group, as described by Warburg and Christian (8). The precipitate obtained after treatment with acid ammonium sulfate was dissolved in water and a little pyrophosphate, 0.2 M, pH 8.3, and used without further purification.

Flavin-adenine-dinucleotide (FAD) was prepared according to Warburg and Christian (8). Its purity was determined spectrographically by comparison with riboflavin. The first preparation was found to be 52 per cent pure, and the second 100 per cent. The latter was used in most of the experiments.

For measurement of enzymatic activity the rate of O_2 uptake with d-alanine as substrate was determined by the usual Warburg technique. Unless otherwise stated each vessel contained in the final reaction mixture 0.05 M dl-alanine[4] and 0.0625 M pyrophosphate, pH 8.2, in a total volume of

[4] In this reaction mixture, the l-alanine neither acts as a substrate nor interferes with the functioning of the d-amino acid oxidase.

3.2 ml. The enzyme, 0.3 to 0.4 ml., was added from the side arm. To the enzyme solution had been added crystalline catalase in an amount sufficient to prevent the appearance of any hydrogen peroxide. Inhibitors and FAD were added in the main compartment to give the indicated final concentration. Solutions of all acid or alkaline substances were adjusted to pH 8.2 before use, except quinine dihydrochloride. This substance did not remain in solution at pH 8.2 in the concentration required for the stock solution; it was adjusted to pH 6, and in the final concentrations under the conditions used, remained in solution and did not alter the pH of the reaction mixtures. For initial experiments the bath temperature was 30°, and the gas phase, air. Later experiments were conducted at 37°. Oxygen was the gas phase for quantitative experiments, while air served for qualitative purposes. The vessels were shaken 10 minutes for temperature equilibration, after which the side arm contents were added, and readings taken at 5 minute intervals, starting 5 minutes after mixing. The rate of O_2 uptake was linear for 30 to 40 minutes and, with excess FAD, was directly proportional to the enzyme concentration for rates less than 150 microliters of O_2 per 20 minutes. An enzyme concentration was usually selected that would give a maximum velocity between 90 and 140 microliters of O_2 per 20 minutes.

For the determination of the dissociation constant of the enzyme-quinine or atabrine compound, the rate with five to six graded concentrations of FAD alone and in the presence of one concentration of inhibitor was measured. Higher concentrations of FAD were used with inhibitor than with controls, selected so that all rates would be readily measurable and would range between 20 and 80 per cent of the maximum. It was impracticable to test in this manner more than two concentrations of inhibitor on 1 day; therefore, in a few experiments several concentrations of inhibitor were tested with only one intermediate FAD concentration. Duplicate experiments performed on the same day checked within 5 per cent, but enzyme solutions sufficiently dilute to give the desired maximum rate at 37° with O_2 as gas phase showed a small but significant loss in activity after 24 hours storage at 0°. This is in contrast to the more concentrated ($\times$ 3 to 4) solutions used at 30°, with air as gas phase, required to attain the same rate. The latter solutions were completely stable for nearly a week at 0°.

Results

It was found that atabrine strongly inhibited d-amino acid oxidase when the concentration of FAD was low, but had little effect in the presence of high concentrations.[5] Similar results were obtained with quinine and numerous other compounds. The results of a few typical experiments are

[5] Compare similar observations of Wright and Sabine (4).

shown in Table I. Also included for comparison are data for three other inhibitors, benzoic acid, *p*-aminobenzoic acid, and 3-diethylamino-7-di-*n*-butylamino-1-methylpbenazthionium chloride,[6] the actions of which are

TABLE I

Effect of Concentration of Flavin-Adenine-Dinucleotide (FAD) on Inhibition of d-Amino Acid Oxidase by Atabrine and Related Compounds

FAD added	Control rate	Inhibitor		Inhibition	Temperature
$\mu \times 10^7$	microliters O_2 per 20 min.		μ	per cent	°C.
1.1	23	Atabrine	0 001	80	30
4.1	99			54	
11.0	150			4	
0.4	35	"	0 001	70	37
2.9	96			36	
27.5	144			8	
0.4	43	Quinine	0 001	61	37
2.9	113			28	
27.5	179			10	
0.4	43	"	0.003	72	37
2.9	113			44	
27.5	179			21	
0.4	43	Plasmochin	0 003	71	37
2.9	113			39	
27.5	179			5	
1.1	28	Sulfanilamide	0 01	65	30
9.4	141			2	
0.6	33	Aniline	0 03	45	30
2.7	90			21	
10.3	131			0	
0.3	23	Benzoic acid	0.0004	64	30
6.4	119			64	
0.3	22	SN8285-4*	0 0004	64	30
6.4	130			58	
0.0	17	*p*-Aminobenzoic acid	0.003	39	37
1.1	90			36	
6.6	142			35	

* 3-Diethylamino-7-di-*n*-butylamino-1-methyl*phenazthionium* chloride (Hellerman, L., Porter, C. C., and Bovarnick, M. R., unpublished data). The Survey number, designated SN, identifies a drug in the records of the Survey of Antimalarial Drugs. The antimalarial activities of those compounds to which Survey numbers have been assigned will be tabulated in a forthcoming monograph.

of a different nature. The phenazthionium dye appears to effect an irreversible inhibition, accompanied by visible precipitation and probably denaturation of the protein. Benzoate and *p*-aminobenzoate indeed

[6] Hellerman, L., Porter, C. C., and Bovarnick, M. R., unpublished data.

participate in reversible competitive inhibition, but the competition is with the substrate rather than the prosthetic group (1)[2] and therefore is unaffected by FAD.

Reversible Inhibition by p-Chloromercuribenzoate—The *d*-amino acid oxidase is susceptible to yet a third kind of reversible inhibition, as typified by its behavior with *p*-chloromercuribenzoate ion.[7] If 0.0001 M potassium *p*-chloromercuribenzoate solution is added to the split enzyme 15 minutes before addition of excess (10^{-6} M) FAD, the activity is reduced to 55 per cent of the control rate. If the FAD has been first added, the rate is 72 per cent of the control. If *p*-chloromercuribenzoate has been added first, followed after 15 minutes by 0.001 M HS-glutathione, and then by FAD, the rate is 97 per cent of the control, glutathione alone being without influence on the rate. The partial protection of the enzyme with respect to *p*-chloromercuribenzoate by FAD added initially is of a different category from the effect of FAD in connection with inhibition by quinine, atabrine, etc. In the latter case a high concentration of FAD prevents inhibition equally effectively when added before, after, or with the inhibitor. Also, as would be anticipated, glutathione is without influence on the action of these compounds. It might at first appear that, since FAD is incapable of reversing the action of *p*-chloromercuribenzoate and its own association with the enzyme is reversible, it should also be incapable of influencing the inhibition by *p*-chloromercuribenzoate. The apparent inconsistency probably resides in a difference in the rate of reaction of *p*-chloromercuribenzoate with enzyme-protein in the presence and in the absence of FAD. FAD combines very rapidly, prolonged incubation leads to no greater activity than is observed immediately after mixing. The reaction between *p*-chloromercuribenzoate and protein is a slower one, the inhibition increasing with the time allowed for reaction between protein and inhibitor. Moreover, the dissociation constant of FAD-protein is exceedingly small, especially at 30°, the reaction temperature; thus in the presence of FAD the concentration of free protein is correspondingly low, and this would be reflected in a greatly decreased rate of reaction with *p*-chloromercuribenzoate under the conditions.

Competitive Inhibition with FAD—For further investigation of competition between prosthetic group and inhibitor, there were included only those compounds that showed nearly complete reversal of inhibition in the presence of high concentrations of FAD, reversal being entirely independent of the order in which the reactants were mixed. In order to summarize the results briefly and in a way that makes possible a semiquantitative

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[7] On the basis of these tests the separated protein might be assumed to possess sulfhydryl character (9), but this has not been completely established. See also the results of Singer and Barron (10)

TABLE II

Inhibition of d-Amino Acid Oxidase by Compounds That Exhibit Competitive Inhibition with Flavin-Adenine-Dinucleotide (FAD)

Inhibitor	$\dfrac{C_Q}{C_I}$ at 37°*		$\dfrac{C_Q}{C_I}$ at 30°
		Range	
Auramine,† 0.0003–0.0009 M	7	3–10	
Atabrine, 0.000006–0.003 M	2.5	2– 5	2
SN12,710-6029,‡ "novalauramine,"§ 0.0003–0.0009 M	2	1– 5	
Quinine, 0.0003–0.003 M	1		1
" methochloride, 0.001–0.003 M	1	0.5 – 1.5	1
6-Methoxyquinoline, 0.001–0.003 M	1	1 – 1	1
Plasmochin, 0.001–0.003 M	1	0.5 – 3	
SN7135, 0.001–0.003 M			1
SN6911, ‖ 0.001–0.003 M	0.4	0.3 – 0.5	0.5
SN7618, ‖ 0.001–0.003 "	0.5	0.3 – 1	0.5
SN1796, 0.0003 M (approximate)			0.07
Sulfathiazole, 0.0003–0.003 M	0.2	0.15– 0.2	0.04
Sulfapyridine, 0.003 M			0.04
Sulfadiazine, 0.003 "			0.015
Sulfanilamide, 0.0003–0.01 M	0.07	0.03– 0.1	0 015
Benzenesulfonamide, 0.003–0.01 M	0.04	0.02 0.1	
SN ,527, ¶ 0.003–0.01 M	0 04		
Aniline, 0.003–0.03 M	0.04	0.03– 0.05	0.01
Pyridine, 0.003–0.09 M	0.02	0.01– 0.03	0.007
dl-p-Dimethylaminophenylaminoacetic acid,** 0.003–0.01 M	0.03 or less		0.007
SN11,526, ††‡‡ 0.01 "	<0.03		<0.007
dl-p-Hydroxyphenylaminoacetic acid‡‡§§			<0.007
4-Diethylamino-1-methylbutylamine‡‡			

* C_Q/C_I, ratio of concentrations of quinine and inhibitor required to give the same degree of inhibition at any one concentration of FAD. For discussion of the reliability of this ratio, see the text.

† (p) $(CH_2).\overset{+}{N}=C_6H_4=C-C_6H_4$ $N(CH_3)_2$ (p').

    Cl^- NH_2
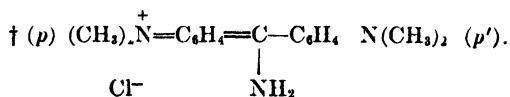
‡ See the foot-note to Table I for the meaning of SN.

§ (p) $(CH_2)_2\overset{+}{N}=C_6H_4=C-C_6H_4-N(CH_3)_2$ (p')

     $HN-CH-(CH_2)_3-\overset{+}{N}(C_2H_5)_2$ (dicitrate)

       CH_3 H
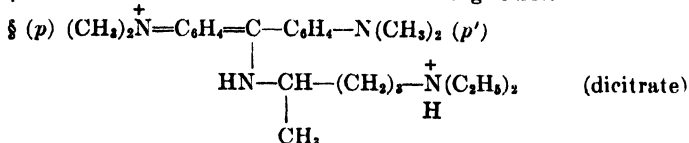
to be described.

‖ Derivatives of 4-aminoquinoline; SN7618 is 4-(4-diethylamino-1-methylbutyl-amino)-7-chloroquinoline (diphosphate)

TABLE II—*Concluded*

¶ N-(4-Diethylamino-1-methylbutyl)-β-(p-dimethylaminophenyl)*alanine,*

$$(CH_3)_2N—C_6H_4—CH_2—CH—COO^-$$
$$|$$
$$HN—CH—(CH_2)_3—\overset{+}{N}H(C_2H_5)_2$$
$$|$$
$$CH_3$$
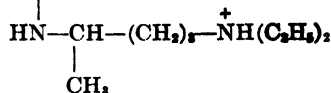
to be described.

** (‖) $(CH_3)_2N—C_6H_4—CH$ COO ; non-substrate for *d*-amino acid oxidase (fo t-
$$|$$
$$NH_3^+$$

note 1)

†† $C_6H_4—CH_2—CH—COO^-$
$$|$$
$$HN—CH—(CH_2)_3—\overset{+}{N}H(C_2H_5)_2$$
$$|$$
$$CH_3$$
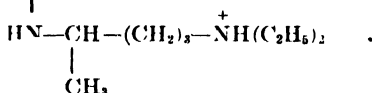
‡‡ No observable inhibition.

§§ See foot-note 1

comparison of the effectiveness of different compounds, all have been compared with quinine as standard. The ratio of the concentration of ·quinine. C_0, to that, C_I, of the second inhibitor required to produce the same rate with the same total FAD and enzyme concentrations should be equal to the ratio of the dissociation constants of the two inhibitor-protein complexes. With inhibitors more effective than quinine, this ratio ($C_0/C_I = K_0/K_I$) is greater than 1; with less effective (more easily dissociated) inhibitors it is less than 1. The wide range in some of the estimated concentration ratios is not due to inconsistency in the experimental results. With any one enzyme preparation and FAD concentration, the degree of inhibition found with each compound was reproducible within the error of the Warburg method (5 per cent). Part of the variation is attributable to the difficulty in estimation of the appropriate quinine concentration, since large (3-fold) changes in quinine concentration lead only to relatively small changes in the amount of inhibition. In addition, not all of the compounds exhibit precisely the same relation between rate and relative concentration of inhibitor and FAD.[8]

Nature of Competitors—It appears thus that a variety of aromatic nitrogen compounds can combine to some extent with the protein portion of *d*-amino acid oxidase in such a way as to prevent combination of the protein with FAD. The most effective substances in this respect contain more than one aromatic ring; *e.g.*, the quinolines, auramines, and atabrine. The ring-substituted sulfonamides, sulfathiazole and sulfapyridine, but not sulfadiazine, are more effective than sulfanilamide. The three compounds listed at the bottom of Table II contain no aromatic nitrogen and give no

[8] Compare the quantitative experiments with atabrine and quinine.

evidence of any inhibition. Slight inhibition was observed with benzene-sulfonamide; at the maximum concentration attainable, 0.01 M, the inhibition with low FAD was only approximately 30 per cent, but since this was reproducible and reversed by increase in FAD it probably is significant. It should be noted that the order of effectiveness of the different inhibitors was very nearly the same at 30° and at 37°. The relative concentration ratios of the different quinolines and atabrine and of the sulfonamides and more weakly associated compounds are also nearly the same. However, at 30° the difference between quinolines and sulfonamides is much greater than at 37°. The change in relative "dissociation constant" at the two temperatures seems too great to be attributable solely to experimental error, and may be related in part to a difference in the heats of dissociation of the two sets of compounds.

Quantitative Evaluation—For conclusive demonstration of the existence of a reversible competition between prosthetic group and inhibitor, the data thus far presented are inadequate, since for this purpose the concentration of free FAD must be known. This is not even approximately equal to the total FAD at the low concentrations, where appreciable inhibition is observed. Its evaluation requires measurement of the rate at a larger number of FAD concentrations. The first attempts at quantitative evaluation of inhibitor and FAD dissociation constants were made at 30°. However, at this temperature the FAD was so tightly bound by the protein that the rate appeared to increase linearly with increase in FAD concentration until it reached a value less than 15 per cent of the maximum; the amount of dissociation for the greater part of the range was apparently lower than the limits of experimental error. At 37°, however, the dissociation was readily detectable.[9] At this temperature there was undertaken a more detailed study of the quantitative aspects of inhibition by quinine and by atabrine.

For the determination of the dissociation constant of an inhibitor that competes with the prosthetic group, account must be taken not only of the equilibria between protein and inhibitor and protein and prosthetic group, but in addition of the equilibrium between substrate and complete enzyme.

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[9] The decrease in the dissociation of FAD with decrease in temperature is further illustrated by certain experiments that were originally undertaken to determine whether reversible dissociation of FAD was characteristic of the complete native enzyme, before it had been subjected to the rather drastic treatment with acid ammonium sulfate. For this purpose FAD sufficient to give the maximum rate at 30° was added to the separated protein and the behavior of this reconstituted enzyme compared with that of the original native enzyme. Precipitation with neutral ammonium sulfate or 3 day dialysis at pH 7.4, both at 0°, led only to a 10 per cent loss of FAD from the reconstituted enzyme, and 5 per cent for the native. Thus at this low temperature each behaved essentially as a non-dissociable enzyme.

The last two already have been studied in detail for d-amino acid oxidase by Stadie and Zapp (11). However, it did not seem justifiable to apply the values found by these authors to data obtained in this laboratory, on the assumption that all preparations of d-amino acid oxidase would show identical dissociation constants, inasmuch as crude enzyme preparations were employed in both investigations.

In the description of the method adopted for calculation of the dissociation constants the following abbreviations have been used. All concentrations are given in moles per liter, velocities in microliters of O_2 per 20 minutes.

p = uncombined enzyme-protein

PF = enzyme-protein combined with FAD

PFS =   "   "   "   " and d-alanine

PI =   "   "   " inhibitor

$P_t = p + PF + PFS + PI$ = total enzyme-protein

F_t = total FAD

f = free FAD

S = d-alanine, free, assumed equal to total

I = inhibitor,   "   "   "   "   "

$(PF)_t = PF + PFS$

V = rate of O_2 uptake

V_{mF} = "   "   "   "   at constant substrate concentration when $F_t = \infty$

V_m = "   "   "   "   when $F = \infty$ and $S = \infty$

$$K_F = \frac{p \cdot f}{(PF)} ; \quad K_S = \frac{(PF)S}{(PFS)} ; \quad K_I = \frac{p \cdot I}{(PI)} ; \quad K_{FS} = \frac{K_F K_S}{K_S + S}$$

In connection with the data at constant substrate concentration it is convenient to use the combined substrate-FAD constant, K_{FS}, which, as will be shown below, is equal to $p \cdot f/(PF)_t$. As always, it is assumed that the velocity is proportional to the concentration of enzyme combined with both prosthetic group and substrate. Then $V = k(PFS)$. When $F = \infty$,

$$P_t = PF + PFS = PFS \left(1 + \frac{K_S}{S} \right) \quad \text{and} \quad V_{mF} = k(PFS) = k \frac{P_t}{1 + \frac{K_S}{S}}$$

$$\frac{V}{V_{mF}} = \frac{PF + PFS}{P_t} \tag{1}$$

$$\frac{V_{mF} - V}{V} \quad \frac{P_t - (PF + PFS)}{PF + PFS} \quad \frac{p}{(PF)_t}$$

$$\frac{V_{mF} - V}{V} \times f = \frac{p \cdot f}{PF(1 + S/K_S)} = \frac{K_F K_S}{S + K_S} = K_{FS} \tag{2}$$

The method described by Haas *et al.* (12) was used for calculation of P_t, which is considered equal to the maximum FAD-combining capacity of the enzyme preparation. These authors ignored the substrate equilibrium in deriving an equation for calculation of P_t, assuming a combination of 1 mole of enzyme with 1 mole of FAD, from the maximum rate (FAD $= \infty$) and the rates at any two intermediate concentrations of FAD. If the substrate equilibrium is included, with the substrate concentration kept constant, $(PF)_t$ used in place of PF, and V_{mF} in place of V_m, the same final equation can be derived.

$$P_t = \frac{F_{t_2}\left(\dfrac{V_{mF} - V_2}{V_2}\right) - F_{t_1}\left(\dfrac{V_{mF} - V_1}{V_1}\right)}{\dfrac{V_1 - V_2}{V_{mF}}} \qquad (3)$$

As mentioned earlier, F_t was slightly larger than the added FAD, since a small amount always remained in the enzyme after treatment with acid ammonium sulfate. The latter quantity was estimated by extrapolation from the FAD-rate curve. V_{mF} was estimated as a first approximation by plotting $1/V$ against $1/F_t$, with only the high values of F_t, where the FAD is largely free, and extrapolating to $1/F_t = 0$. After calculation of P_t and f with this value of V_{mF}, a closer approximation to the true value of $1/V_{mF}$ was made by plotting $1/V$ against $1/f$. This second value of V_{mF} generally gave values for P_t and K_{FS} that showed no drift. If this was not so, V_{mF} was finally adjusted by trial and error, since the change necessary was never greater than 1 to 2 per cent and not graphically detectable.

After estimation of P_t, the combined FAD could be calculated readily by Equation 1 and the free FAD from the difference between total and combined. K_{FS} was then given by Equation 2.

When inhibitor was present, the values of P_t, K_{FS}, and V_{mF} found in the corresponding control experiment were used to calculate the concentration of free FAD, free protein, inhibitor-bound protein, and finally inhibitor dissociation constant from the following relationships.

$$(PF)_t = \frac{V}{V_{mF}} \times P_t; \quad f = F_t - (PF)_t; \quad p = K_{FS} \times \frac{(PF)_t}{f}; \quad K_I = \frac{p \cdot I}{(PI)}$$

In order to be able to compare inhibitor and prosthetic group dissociation constants, it was necessary to know the value of K_S. In a few experiments V_{mF} was estimated at several different substrate concentrations from values of V at two or three relatively high FAD concentrations.

From the intercept of the $1/V_{mF}$ *versus* $1/S$ line, $1/V_m$ could be determined. The substrate dissociation constant is then given by the equation

$$\frac{V_m - V_{mF}}{V_{mF}} \times S = K_S$$

K_F can then be calculated from K_{FS} by Equation 2.

Dissociation Constants—The results and detailed calculations for two control experiments are given in Table III. The data obviously conform well to the relationship predicted for a reversibly dissociable enzyme-prosthetic group system, as indicated by the constancy of the values of K_{FS}.

Typical data for calculation of inhibitor dissociation constants are given in Table IV. The values calculated for the quinine-protein dissociation constant, although less consistent than those for K_{FS}, showed no marked drift with change in FAD or quinine concentration. This is far from true in the case of atabrine. The calculated dissociation constants for atabrine showed a marked upward drift with increase in FAD or in atabrine concentration.

The results of all measurements of FAD, substrate, quinine, and atabrine dissociation constants are summarized in Table V. Although in each individual experiment there was good agreement between the dissociation constants found with different amounts of FAD, the constants with different enzyme preparations varied. With preparations made from the same lot of lamb kidneys, FAD and quinine dissociation constants agreed within 10 per cent. However, with preparations from different lots of lamb kidneys the divergence in both constants and in the values of P_t was marked. This variation may be caused in part by use of a crude enzyme preparation, for there are several flavoproteins in tissue and certain of these can be split reversibly by acid ammonium sulfate (13). The presence of one or more such proteins with FAD dissociation constants not too far removed from that of d-amino acid oxidase might alter the magnitude of K_{FS} without causing the system, within the limits of experimental error, to depart from the velocity-concentration relationship of a single dissociating system.

The value of K_Q would also be influenced by contaminating FAD-combining proteins, but not by other types of foreign proteins capable of combination with quinine, since the relatively high quinine concentrations used would not be altered significantly by such combination.

There is fairly good agreement between the K_S values found with the two lots of kidneys. This constant should be less affected by foreign proteins than K_F or K_Q, inasmuch as proteins other than d-amino acid oxidase itself probably would not combine with d-alanine.

The values for K_F found with Enzymes I-32, I-33, and I-34 (average,

TABLE III

Calculation of K_{FS} for d-Amino Acid Oxidase (37°)

Enzyme No.	P added	V	$\dfrac{V_{mP}-V}{V}$	F_t	$F_t\dfrac{V_{mP}-V}{V}$	$\dfrac{V}{V_{mP}}$	P_t (1)*	P_t (2)*	$(PF)_t$	f	K_{FS}
	$M \times 10^7$	microliters O$_2$ per 20 min.		$M \times 10^7$	$M \times 10^7$		$M \times 10^7$	$M \times 10^7$	$M \times 10^7$	$M \times 10^7$	moles per l $\times 10^7$
II-43	0	16.6	6.84	0.34†	2.32	0 127	1.18‡	1.02‡	0.108	0.23	1.58‡
	0.509	37.3	2.49	0.85	2.11	0.286	1.01	0.87	0.248	0.60	1.50
	1.02	52.7	1.47	1.36	1.99	0.404			0.350	1.01	1.48
	2.03	72.7	0.791	2.37	1.88	0.558	0.75		0.482	1.89	1.50
	4.07	93.3	0.395	4.41	1.74	0.716	0.80	0.86	0.621	3.79	1.50
	7.47	107.5	0.211	7.81	1 65	0.825	0.82	0.87	0.716	7.09	1.50
	27.1	123.2	0.0568	27.5		0 946			0.821	26.7	1.51‡
	∞	130.2†									
Average								0.86			1.49
II-41	0	15.2	8.26	0.305†	2.52	0.108	1.32‡	1.28‡	0.129	0.18	1.46‡
	0.55	38.3	2.675	0.855	2.29	0.272	1 15	1 18	0.322	0.53	1.43
	1.10	55.8	1.523	1.41	2.14	0.396	1 21		0.468	0.94	1.43
	2.21	79.4	0.773	2.51	1.94	0.564			0.669	1.84	1.42
	4.14	100.7	0.398	4.45	1.77	0.716	1.17	1.12	0.849	3.60	1.43
	8.28	118.7	0.186	8.59	1.60	0.843	1 22	1.23	0.998	7.59	1.41
	27.6	133.6	0.0539	27.9		0.949			1.12	26 8	1.44‡
	∞	140.8†									
Average								1 18			1.42

* P_t (1), calculated by comparison of each point with the one for which F_t = 1.36 (Enzyme II-43) or 1.41 (Enzyme II-41) by Equation 3. P_t (2), calculated by comparison of each point with the one for which F_t = 2.37 (Enzyme II-43) or 2.51 (Enzyme II-41). The functions, $F_t(V_{mF} - V)/V$ and V/V_{mF}, were calculated from the rate at each value of F_t. The most reliable values of these functions were in the range of flavin-adenine-dinucleotide (FAD) concentration in which the velocity was approximately one-half of the maximum. Therefore one such point was selected for F_{t1} and V_1, and P_t was calculated with each other point in turn as F_{t2} and V_2 by Equation 3. This process was repeated with a second point in the reliable range as standard and all values were averaged to obtain P_t for the particular enzyme.

† Estimated graphically.

‡ Omitted from the average; these points were considered unreliable and ignored whether or not the constants agreed with those calculated from the other points, for the values from the first points (no added FAD) were determined largely by the estimated value of F_t and those from the last point (highest FAD) by the estimated V_{mF}.

0.46 × 10⁻⁶ mole per liter) are in good agreement with that found by Stadie and Zapp (11), 0.47 × 10⁻⁶ mole per liter. Stadie and Zapp's

value for K_S, 0.0053 mole per liter, is somewhat lower than those in Table V. However, later in their paper they give as 0.009 mole per liter the value for the constants K_{PFZS} and K_{PFAS}, which should be equal to K_S.

TABLE IV

Calculation of Protein-Inhibitor Dissociation Constants (37°)

The enzyme used with quinine was Enzyme II-43, $V_{mF} = 130.2$ microliters of O_2 per 20 minutes, $P_t = 0.86 \times 10^{-7}$ M, $K_{FS} = 1.49 \times 10^{-7}$ M, flavin-adenine-dinucleotide (FAD) present in enzyme $= 0.34 \times 10^{-7}$ M. The enzyme used with atabrine was Enzyme II-41, $V_{mF} = 140.8$ microliters of O_2 per 20 minutes, $P_t = 1.18 \times 10^{-7}$ M, $K_{FS} = 1.42 \times 10^{-7}$ M, FAD present in enzyme $= 0.305 \times 10^{-7}$ M

Inhibitor		FAD added	I	F_t	$(PF)_t$	f	p	PI	K_I
	M	$M \times 10^7$	microliters O_2 per 20 min	$M \times 10$	$M \times 10^7$	$M \times 10^7$	$M \times 10^7$	$M \times 10^7$	moles per $l \times 10^2$
Quinine	0 003	2 03	36 5	2 37	0 242	2 13	0 170	0 453	1 12
		4 07	56 2	4 41	0 374	4 04	0 138	0 353	1 17
		8 14	71 3	8 48	0 474	8 01	0 088	0 303	0 87
		13 57	84 6	13 91	0 563	13 35	0 063	0 239	0 79
Average									0 99
Quinine	0 001	1 01	32 0	1 35	0 212	1 14	0 277	0 376	0 74
		2 04	51 1	2 38	0 339	2 04	0 249	0 277	0 90
		4 07	76 6	4 41	0 510	3 90	0 195	0 160	1 21
		8 14	95 8	8 48	0 637	7 84	0 121	0 107	1 13
Average									0 99
Atabrine	0 0003	0 689	22 0	0 995	0 185	0 81	0 327	0 672	0 15
		1 65	48 4	1 96	0 407	1 55	0 374	0 403	0 28
		3 31	79 8	3 61	0 672	2 94	0 325	0 187	0 52
		8 28	109 6	8 59	0 923	7 67	0 172	0 089	0 58
Average									0 38
Atabrine	0 0001	0 550	18 8	0 855	0 158	0 70	0 323	0 703	0 046
		1 10	36 6	1 41	0 308	1 10	0 399	0 477	0 084
		2 20	67 4	2.51	0 571	1 94	0 419	0 194	0.22
		4 14	91 4	4 45	0 770	3 68	0 298	0 116	0 26
		8 28	109 6	8 59	0 923	7 67	0 171	0 090	0 19
Average									0 16

Thus apparently within the limits of variability of both sets of experiments, the results reported in the two investigations are in agreement

The atabrine data, included in Table V, can be taken as an indication that atabrine is effective as an inhibitor at lower concentrations than is

quinine. The cause of the drift in the calculated atabrine dissociation constants is not entirely apparent. Combination of more than 1 mole of atabrine per mole of protein would cause drifts in the reverse direction to those observed. An irreversible action should not be affected by FAD.

<div align="center">TABLE V</div>

<div align="center">*Dissociation Constants of d-Amino Acid Oxidase (37°)*</div>

Enzyme No.*	V_{mF}	P_t	K_{FS}	K_S	K_F	Quinine			Atabrine	
						Concentration	K_Q	$\frac{K_Q}{K_F}$	Concentration	K_A
	micro-liters O₂ per 20 min	moles per l X 10⁷	moles per l. X 10⁷	mole per l.	moles per l. X 10⁷	mole per l	moles per l. X 10⁴		mole per l	moles per l X 10⁴
I-32	110	2.7	0 91	0.0061	4.5	0.003	5 4	1200		
						0.001	4.8	1100		
						0.0003	3 8	800		
						0.0001	5.4	1200		
I-33	96	3.3	0.82		4.0	0.001	5 1	1300		
I-33A	88	2.7	0 90		4 4	0 003	4.3	1000		
						0.001	4.3	1000		
						0 0003	3 5	800		
I-34	124	2.6	1 07		5 3				0 00002	0 47
									0 0002	1.7
									0 0006	8 7
Average					4.6 ± 0.4		4.6 ± 0 6	1000		
II-37	141	0 97	1.33		6.6	0.001	9.2	1400		
II-41	141	1.18	1.42	0.0066	7.1				0.0001	1.6
									0.0003	3.8
II-42	134	1.15	1.30		6.4	0.001	8.6	1300		
						0.0003	7.4	1200		
II-43	130	0.86	1 49		7.4	0.003	9.9	1300		
						0.001	9.9	1300		
Average	....				6.9 ± 0.4		8.9 ± 0.7	1300		

* Enzymes I-32 through I-34 were prepared from one lot of acetone-dried lamb kidneys; the remainder, from a second lot.

The effect of FAD (but not atabrine) concentration on the rate might be explained by the assumption that with atabrine there are two types of inhibition, the one rapid, reversible, and competitive, and the other slower and irreversible, but also affecting only the free protein. Thus increase in FAD would not only lower the magnitude of the first kind of inhibition, but would decrease the rate of the second. This is supported by the fact

that at low FAD concentrations the rate of O_2 uptake was linear only for the first 15 to 20 minutes, and then progressively declined. That the quinine effect also might be associated with a measure of irreversible inactivation was indicated in a few experiments in which the rate was followed for longer than the usual 30 minute period. Although in the longer interval even the control rates ceased to be completely linear, nevertheless the decrease was more marked in systems containing quinine when the FAD concentration was low.

In addition, these observations raise a question that is always troublesome in attempts to derive thermodynamic constants from kinetic data; namely, whether true equilibrium actually has been attained. Obviously the above data are significant only if the equilibria between enzyme, prosthetic group, and inhibitor are attained rapidly. It may be said only that the constant rates usually observed for 30 minutes, starting 5 to 10 minutes after mixing, indicate that the concentration of FAD-enzyme-substrate complex, which must be the resultant of all three equilibria, becomes rapidly constant. If feasible, a longer observation period would be desirable, but unfortunately this is accompanied by a change in substrate concentration and by a slow decline in enzymatic activity. Prolongation of the period of observation thus would offer no advantage.

With any oxidizable enzyme there is an additional factor that has been thus far completely ignored; viz., the difference in the dissociation constants of the oxidized and reduced prosthetic group. The relative oxidation-reduction potentials of the "old yellow enzyme" and of riboflavin phosphate, E_0', at pH 7 amount to -0.06 and -0.18 volt respectively (14), indicating that in this flavoprotein the reduced prosthetic group is some 10^3 times as tightly bound as is the oxidized. Warburg and Christian (8) present qualitative evidence that with d-amino acid oxidase the dissociation of the reduced FAD is very significantly lower than the oxidized. Data obtained with the first set of enzyme preparations (Enzymes I-32 to I-34) with air in the gas phase gave dissociation constants (K_F) for FAD-protein of 2.7 to 3.0×10^{-7} mole per liter as compared to the values 4.0 to 5.3×10^{-7} found with oxygen as the gas phase (Table V). That the increase is no greater than this when the O_2 concentration is increased 5-fold is an indication that FAD exists largely in the oxidized form, even in air.

In view of the possibly complicating and insufficiently controlled factors discussed above, it is surprising that the d-amino acid oxidase system approximates so closely the theoretical behavior of a single enzyme, combining reversibly with prosthetic group, FAD, and substrate. For evaluation of more reliable constants, it would be necessary to work with pure enzyme preparations and to take into quantitative consideration the state of oxidation of FAD.

In spite of the limitations in the significance of the absolute values found for K_F and K_Q, it seems allowable to conclude that, over a limited period of time, quinine inhibits *d*-amino acid oxidase by reversible competition with FAD. Relatively high concentrations of quinine are required, in view of its relatively large dissociation constant. With atabrine there may be reversible competitive inhibition, but the final effect of atabrine, even over a relatively short period, cannot be explained on the single assumption of *reversible* combination with the protein portion of the enzyme.

<div align="center">DISCUSSION</div>

It is apparent from the foregoing that the FAD-inhibitor antagonism is not at all specific for atabrine.[10] It is nearly as marked with quinine, plasmochin, etc., and the non-antimalarial quinolines, quinine methochloride and 6-methoxyquinoline, and is readily detectable with a variety of other simpler aromatic nitrogen compounds in higher concentrations. All of these compounds appear to share the property of combining with numerous proteins. When the protein is an enzyme, the combination may or may not result in inhibition, depending upon whether the portion of the protein molecule affected is essential for activity. When the grouping affected takes part in the combination between enzyme and prosthetic group, the result is competitive inhibition. However, if it is essential for activity, but not for combination between enzyme and substrate or prosthetic group, a non-competitive inhibition results. The latter was found to be true of the inhibition of Straub's (15) flavoprotein (a "coenzyme factor") from heart muscle by some of these compounds.[11]

All of the quinoline compounds and atabrine, and possibly the other basic inhibitors also, appear to combine with similar groups in proteins. In the studies with other enzymes, *e.g.* heart flavoprotein, lactic dehydrogenase, pancreatic lipase, catalase,[11] it was found generally that an enzyme affected by one was affected by all, the order of effectiveness being similar to that found with *d*-amino acid oxidase. In view of the results of the quantitative measurements of atabrine inhibition, it is probable that many of these compounds have in addition to a rapid reversible effect, a slower,

[10] The observations with isolated enzymes thus lend little general support to the idea that a similarity in structure between atabrine and isoalloxazine derivatives is definitive for the chemotherapeutic action. It is of interest in this connection that studies (2) with a particular strain of surviving, isolated parasites (*Plasmodium lophurae*) gave no indication of alteration of the effect of atabrine by flavin nucleotides. It remains, however, conceivable that cells may exist in which the respiration is concerned with the functioning of sufficiently dissociable flavoenzymes, permitting inhibition by atabrine, quinine, sulfathiazole, etc., through a competitive process akin to that treated in this paper.

[11] Unpublished results of the authors.

irreversible one. The irreversible phase is not necessarily marked with all proteins. Thus inhibition of the action of pancreatic lipase by atabrine, as well as by quinine, followed the concentration-rate relationship of inhibition by *reversible* combination. On the other hand, with cytochrome reductase, the irreversible action of atabrine was reported as being so marked (5) that protection by riboflavin phosphate was possible only when this was added before the atabrine. Thus the relative importance of the reversible and irreversible actions of these compounds may vary both with the enzyme and with the inhibitor, as illustrated by the difference between atabrine and quinine in their effect upon *d*-amino acid oxidase.

SUMMARY

1. Quinine, atabrine, and other related substances inhibit *d*-amino acid oxidase strongly at low concentrations of flavin-adenine-dinucleotide (FAD), and only slightly at high concentrations. This suggests inhibition by competition with FAD for the protein. Qualitative comparison of the variation of the degree of inhibition as a function of the concentration of FAD and inhibitor made possible an estimation of the relative affinity of a series of compounds for the protein constituent of *d*-amino acid oxidase. Atabrine and two auramines were the most effective inhibitors, followed by quinine, plasmochin, and other quinoline derivatives. Less effective were the sulfonamides, aniline, and pyridine.

2. There was conducted with quinine and atabrine a more detailed study of the protein-inhibitor-FAD equilibrium. The rate-concentration relationship with quinine as inhibitor was interpretable readily on the assumption of reversible competitive inhibition. This was not strictly true with atabrine. With enzyme preparations from one lot of lamb kidneys the following values were found for the respective dissociation constants of FAD, quinine, and substrate, K_F 4.6×10^{-7} mole per liter, K_S 0.0061 mole per liter, K_Q 4.6×10^{-4} mole per liter. With preparations from a second lot the values were K_F 6.9×10^{-7}, K_S 0.0066, K_Q 8.9×10^{-4} mole per liter.

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LETTERS TO THE EDITORS

A CRYSTALLINE PROTEIN WITH β-AMYLASE ACTIVITY, PREPARED FROM SWEET POTATOES*

Sirs·

A crystalline protein, highly active in splitting reducing sugar from starch, and quite free from α-amylase activity, has been prepared from sweet potatoes.

The press-juice of sweet potatoes was brought to 60°, immediately cooled, and filtered after the addition of lead acetate; the protein in the filtrate was precipitated by 0.7 saturation with ammonium sulfate, and than purified by dialysis, acidification successively to pH 4 6, 4.0, and 3.2, and finally by quarter saturation with ammonium sulfate at pH 3.5 to 4.0, each time with the removal of any precipitate. Repeated fractionation

Preparation	No. of crystallizations	Specific activity
D*	3	1029
	4	1142
E	2	1141
	3	1260
F	3	1235
	5	1070

* The partly purified protein was in this case also submitted to electrodialysis.

of the remaining material between quarter and half saturation with ammonium sulfate at pH 4.0 finally yielded crystals on the cautious addition of ammonium sulfate in the cold. Recrystallization was carried out in the same manner from solutions containing 1 to 2 per cent of protein.

Dialysis of the twice recrystallized substance did not significantly change the nitrogen precipitable by trichloroacetic acid. After dialysis, 17.48 per cent of the dry weight was nitrogen and 17.13 per cent was nitrogen precipitated by trichloroacetic acid.

The amylolytic activity expressed in arbitrary units per mg. of protein nitrogen is given in the table.

* Enzyme Research Laboratory Contribution No. 99.

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The crystals are tetragonal, and usually 12-sided, consisting of short rectangular prisms (generally cubes) with tetrahedral pyramids at each end. They have frequently measured 10 to 15 μ.

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THE ACTION OF METMYOGLOBIN, OXYGEN, AND MANGANESE ON OXALACETIC ACID*

Sirs:

In a previous communication[1] we have reported the presence in animal tissues of a substance capable, when supplemented with manganese ions, of causing an oxidative decarboxylation of oxalacetic acid to malonic acid and carbon dioxide. The factor in heart muscle has been found to be metmyoglobin. This substance, prepared from horse heart[2] and

Effect of Catalase and Hydrogen Peroxide on Oxidation of Oxalacetate in Presence of Mn^{++} and Metmyoglobin

Conditions, gas phase, air; 30°. Experiment I, 0.25 M acetate buffer, pH 4.9, 0.3 mg. of metmyoglobin, 1.5 mg. of oxalacetic acid in a total final volume of 2.0 cc. In (a) 0.1 cc. of 0.01 M $MnCl_2$ and 0.1 cc. of 0.003 N H_2O_2 were tipped in from the side arm at 0 time. In (b) 0.1 cc. of 0.01 M $MnCl_2$ was tipped in from the side arm at 0 time. Experiment II, 0.05 M acetate buffer, pH 4.9, 0.0005 M $MnCl_2$, 3.0 mg. of metmyoglobin in a final volume of 2.0 cc. In (a) no catalase; in (b) 0.3 mg. of crystalline beef liver catalase. 1.5 mg. of oxalacetic acid were tipped in from the side arm at 0 time.

eriment No	Lag period*	Gas exchange		
		Time	O_2 consumed	CO_2 evolved
	m·n	*min.*	*microliters*	*microliters*
H_2O_2	3	18	41	85
		60	69†	166†
No H_2O_2	15	18	6	34
		60	70†	169†
No catalase	10	60	104‡	256‡
Catalase	35	60	11	160

* The lag period is the time when first measurable oxygen consumption can be detected.

† Oxygen consumption is complete at this time; CO_2 evolution continues.

‡ Both CO_2 evolution and oxygen consumption are complete.

recrystallized four times, accounted for the activity of the fresh dialyzed water extracts of the heart muscle. Hemoglobin and cytochrome c were without activity.

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There is an initial lag period before oxygen consumption sets in. This lag period is reduced by the addition of small amounts of hydrogen peroxide and increased by the presence of catalase, as shown in the table.

Under conditions similar to those described in the table, 0.0C03 M sodium azide doubles the lag period and decreases the total oxygen consumption 50 per cent, and 0.003 M sodium azide inhibits oxygen consumption completely. The oxygen consumption, when plotted against time, always gives an S-shaped curve, and if the amount of metmyoglobin is limited as in Experiment I oxygen consumption ceases before all the added oxalacetate has been decomposed. The proportion of oxalacetate oxidatively decarboxylated as compared to that non-oxidatively decarboxylated varies with buffer concentration, pH, and the concentration of the reacting components.

During the oxidation the metmyoglobin is converted first to a green pigment and then to a light brown pigment which shows no sharp absorption bands in the visible spectrum and is inactive in the test system.

There is an analogy in this reaction to the action of peroxidase on dioxymaleic acid,[3] to the coupled oxidation of ascorbic acid and hemoglobin,[4] and particularly to the action of cytochrome c with manganese on dioxymaleic acid.[5] The recent studies by Virtanen and Laine[6] on the hemoglobin and related pigments in root nodules of legumes, with the demonstration of a possible functional relationship of these substances to oxalacetic acid in connection with nitrogen fixation, have prompted the publication of these findings at the present time.

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THE AEROBIC GLYCOLYSIS OF AVIAN RED BLOOD CELLS AND ITS CONTROL BY INTRACELLULAR IONS IN PHYSIOLOGICAL CONCENTRATIONS*

Sirs:

Hemolysates of washed red blood cells of pigeon (1 part of red cells + 2 parts of distilled H_2O), separated from the main bulk of white cells, show a strong glycolysis. It is essentially aerobic, being inhibited 85 to 90 per cent by 0.002 M HCN and 60 to 70 per cent after evacuation of oxygen with the oil pump. This inhibition is irreversible after 3 hours anaerobiosis and partly reversible after $1\frac{1}{2}$ hours.

This aerobic glycolysis, the mechanism of which (perhaps identical with that described for kidney extracts[1]) will be investigated, shows a characteristic sensitivity to multivalent ions which is manifested by strong inhibitory effects varying in degree with the valency and the nature of the ion. These effects are characterized by four features, which are held significant in so far as potential physiological importance and physicochemical mechanism of inhibition are concerned. (1) The inhibitory effect increases with decreasing pH (between 7.6 and 6.7). (2) When multivalent cations are applied together with multivalent or strongly adsorbable univalent anions (SCN^-, PO_3^-), the resulting inhibition is greater than that corresponding to simple summation. This synergy between cations and anions is particularly marked with Mg^{++} which by itself is a specific activator of glycolysis up to 0.005 M and inhibits only in higher concentrations. Mg^{++}, however, strongly increases the inhibition by multivalent (*e.g.* phosphate) and strongly adsorbable anions even below 0.005 M. (3) Ions, which are normal cell constituents, inhibit strongly in physiological concentration (Ca at 0.00025 M, inositol hexaphosphate, ribonucleate at 0.001 M, phosphate at M/65 in the presence of 0.0025 M Mg^{++}). (4) The inhibition is reversible (so far investigated only for calcium).

Slices of salivary glands, pancreas, and liver, exposed to stimulating agents (pilocarpine, acetylcholine, secretin),[2] and brain slices suspended in 0.16 M NaCl solution show great increase in respiration and simultaneously aerobic glycolysis.[3] These effects are transitory and reversible and therefore not an expression of cell damage but of stimulation. They are irre-

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Influence of Ions on Aerobic Glycolysis

1 part of red blood cell suspension + 2 parts of distilled H_2O; time 200 minutes, temperature 24°.

Added substance	Lactic acid formed from glucose per 100 ml. cell suspension	Inhibition
	mg.	*per cent*
Glucose, 0.18%, pH 7.1..	60	
" + 0.00025 M CaCl₂.........	46	24
" + 0.01 M Na₂SO₄........................	57	5
" + 0.00025 M CaCl₂ + 0.01 M Na₂SO₄...........	17	71
" + 0.001 M inositol hexaphosphate..............	39	35
" + 0.001 " " " + 0.00025 M CaCl₂..:.............	16	73
Glucose + 0.0025 M MgCl₂............................	78	0
" + 0.0025 " " + 0.01 M Na₂SO₄........... ..	53	31
" + M/1300 ribonucleate........................	52	13

versibly suppressed by prolonged anaerobiosis. It seems possible that this "stimulation metabolism" is closely related to the aerobic glycolysis of nucleated red blood cells. The responsiveness of the latter to changes of the ionic environment supports this view.

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INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

XXVI. THE DETERMINATION OF METHIONINE IN PROTEIN HYDROLYSATES WITH LACTOBACILLUS FERMENTI 36*

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It has been shown that methionine is essential or is accessory for the satisfactory growth of *Lactobacillus casei* (2), *Lactobacillus arabinosus* 17-5 (3–8), *Leuconostoc mesenteroides* P-60 (9), and *Lactobacillus fermenti* 36 (10), and the determination with *Lactobacillus arabinosus* of methionine in a mixture containing eleven amino acids has been described by Shankman, Dunn, and Rubin (4). The determination of methionine in protein hydrolysates with *Lactobacillus fermenti* 36, *Leuconostoc mesenteroides* P-60, and *Lactobacillus arabinosus* 17-5 is described in this paper.

EXPERIMENTAL

The assay technique described in Paper XXV (1) was employed. The following basal media were utilized for the growth of the microorganisms: *Lactobacillus fermenti*, that given in Paper XXIV (10), *Leuconostoc mesenteroides*, essentially that given in Paper XVIII (9), and *Lactobacillus arabinosus*, essentially that given by Shankman (3). The total volume in each tube (4 inch) was 3 ml. All of the solutions were adjusted to the same concentration of sodium chloride to compensate for any stimulatory or inhibitory salt effects. The standard was run at fourteen levels up to 28 γ of the amino acid, the amino acid test mixtures and protein hydrolysates were run at five levels, and six tubes were employed at each level of sample and standard. The contents of the six tubes at each level were mixed and titrated and the average titration value was calculated from these data.

It was found that $d(+)$-methionine was completely inactive in promoting growth, either of *Leuconostoc mesenteroides* or *Lactobacillus arabinosus*. On the other hand, it stimulated the growth of *Lactobacillus fermenti* to an

extent entirely comparable to that exhibited by the $l(-)$ antipode. It appears that this response of *Lactobacillus fermenti* to both optical isomers

TABLE I

Assay of Methionine in Amino Acid Test Mixture 1* (dl-Methionine Content 4.81 Per Cent)

Amino acid mixture per tube	dl-Methionine present per tube	Titration volume†	dl-Methionine found per tube	dl-Methionine recovered
γ	γ	ml.	γ	per cent
84.4	4.06	4.09	4.11	101.2
168.8	8.12	6.70	8.00	98.5
253.1	12.18	8.40	12.40	101.8
337.5	16.24	10.02	16.29	100.3
421.9	20.30	11.81	20.40	100.5
Average..				100.5

* The composition of the test mixture simulating that of casein was the same as that given in a previous paper (1). The basal medium (Medium C, Table I, Paper XXIV (10)) and 2 day incubation at 35° with *Lactobacillus fermenti* were employed.

† The solutions of six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

TABLE II

Assay of Methionine in Amino Acid Test Mixture 2* (dl-Methionine Content 0.42 Per Cent)

Amino acid mixture per tube	dl-Methionine present per tube	Titration volume†	dl-Methionine found per tube	dl-Methionine recovered
γ	γ	ml.	γ	per cent
960	4.00	4.01	4.00	100.0
1920	8.00	6.61	7.89	98.6
2880	12.00	8.50	12.62	105.2
3840	16.00	9.92	16.06	100.4
4800	20.00	11.59	19.82	99.1
Average..				100.7

* The composition of the test mixture was the same as that given in a previous paper (1). The basal medium (Medium C, Table I (10)) and 2 day incubation with *Lactobacillus fermenti* were employed.

† The contents of six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

of an amino acid is a unique property of microorganisms not hitherto recorded in the literature.[1] The titrations at fourteen levels of methionine

[1] It has been reported by Dunn et al. (11), Hac (quoted by Lyman et al. (12)), Lyman et al. (12), Lewis and Olcott (13), and Hac et al. (14) that $d(-)$-glutamic acid

TABLE III

Assay of Methionine in Amino Acid Test Mixture 3* (dl-Methionine
Content 1.65 Per Cent)

Amino acid mixture per tube	dl-Methionine present per tube	Titration volume†	dl-Methionine found per tube	dl-Methionine recovered
γ	γ	ml.	γ	per cent
125.3	2.07	3.72	1.96	94.7
250.6	4.14	5.49	4.20	101.5
376.0	6.21	7.81	6.35	102.3
501.3	8.29	9.90	8.25	99.5
626.6	10.36	12.20	10.54	101.7
Average..				99.9

* The composition of the test mixture simulating that of silk fibroin was the same as that given in a previous paper (1). The basal medium (Medium C, Table I (10)) and 2 day incubation with *Lactobacillus fermenti* were employed.

† The solutions of six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

TABLE IV

Assay of Methionine in Casein Hydrolysate*

Hydrolyzed casein per tube (moisture- and ash-free)	Titration volume†	Methionine found per tube	Methionine in casein
γ	ml.	γ	per cent
149.2	4.25	4.40	2.95
298.4	7.31	9.40	3.15
447.6	9.10	14.00	3.13
596.7	10.68	17.72	2.97
745.9	12.40	21.90	2.93
Average..			3.03

* The basal medium (Medium C, Table I (10)) and 2 day incubation with *Lactobacillus fermenti* were employed.

† The solutions of six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

in tubes containing from 2 to 28 γ of methionine per tube averaged 3.5 per cent higher volumes of standard base for the *dl-* than for the *l*(−)-methio-

promoted the growth of *Lactobacillus arabinosus*, although the *d*(−) antipode approached activity equivalent to that of the *l*(+) isomer only at high concentration. Stokes and Gunness (15) found that *d*(−)-aspartic acid exhibited essentially the same type of activity in promoting the growth of *Lactobacillus delbrückii*.

nine. ' Since an analytically pure grade of *dl*-methionine was employed, it appears probable that the *l*(−)-methionine (obtained through the courtesy of the A. E. Staley Manufacturing Company, Decatur, Illinois)

TABLE V

Recovery of Methionine Added to Casein Hydrolysate*

Hydrolyzed casein per tube (moisture- and ash-free)	Methionine			
	In casein per tube†	Added per tube	Found per tube	Recovered
γ	γ	γ	γ	*per cent*
74.6	2.26	2.00	4.30	102.0
149.2	4.52	4.00	8.50	99.5.
223.8	6.78	6.00	13.05	104.5
298.4	9.04	8.00	16.92	98.5
373.0	11.30	10.00	20.95	96.5
Average...				100.2

* The sample was prepared to contain 10.0 γ of methionine per ml. and 373.0 γ of moisture- and ash-free casein per ml.   Volumes from 0.20 to 1.00 ml. were taken for the assays.   The basal medium (Medium C, Table I (10)) and 2 day incubation with *Lactobacillus fermenti* were employed.

† Estimated on the basis of the 3.03 per cent of methionine in casein given in Table IV.

TABLE VI

Assay of Methionine in Silk Fibroin Hydrolysate*

Hydrolyzed silk fibroin per tube (moisture- and ash-free)	Titration volume†	Methionine found per tube	Methionine in silk fibroin
γ	*ml.*	γ	*per cent*
1881	3.90	2.80	0.149
3763	4.81	5.10	0.136
5644	5.91	7.75	0.137
7526	7.30	10.48	0.139
9407	9.02	13.30	0.141
Average..			0.140

* The basal medium (Medium C, Table I (10)) and 2 day incubation with *Lactobacillus fermenti* were employed.

† The solutions of the six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

contained several per cent of inactive material.   The titrations in the experiments with *Leuconostoc mesenteroides* at fourteen levels of methionine in tubes containing from 1 to 14 γ of methionine per tube were the same

(within ±1.1 per cent) for dl-methionine and half the quantities of l(−)-methionine. The titrations in the experiments with *Lactobacillus arabinosus* at ten levels of methionine in tubes containing from 1 to 12 γ of methionine per tube averaged 2.2 per cent higher volumes of standard base for the dl- than for half the quantity of l(−)-methionine.

The casein, the silk fibroin, and the hydrolysis procedure were the same as those described in Paper XVII (11). The experimental data are given in Tables I to X.

TABLE VII

Recovery of Methionine Added to Silk Fibroin Hydrolysate[*]

Hydrolyzed silk fibroin per tube (moisture- and ash-free)	Methionine			
	In silk fibroin per tube[†]	Added per tube	Found per tube	Recovered
γ	γ	γ	γ	*per cent*
941	1.32	2.00	3.50	109.0
1881	2.63	4.00	6.72	102.2
2822	3.95	6.00	9.60	94.2
3763	5.27	8.00	13.40	101.6
4704	6.58	10.00	15.88	93.0
Average...........				100.0

[*] The sample was prepared to contain 10.0 γ of methionine and 4704 γ of moisture- and ash-free silk fibroin per ml. Volumes from 0.20 to 1.00 ml. were taken for the assays. The basal medium (Medium C, Table I (10)) and 2 day incubation with *Lactobacillus fermenti* were employed.

[†] Estimated on the basis of the 0.140 per cent of methionine in silk fibroin given in Table VI.

DISCUSSION

It has been found that methionine could be determined most accurately by incubating *Lactobacillus fermenti* 36 inoculum (diluted with a volume of sterile saline 3 to 9 times that of the medium from which it was centrifuged) for 2 days at 35° in a basal medium of the composition of Medium C, given in Table I, Paper XXIV (10). Under these conditions, the average mean deviation from the mean values (all mean values at five levels of each sample) was about 2.5 per cent for three amino acid test mixtures which contained 4.81, 1.65, and 0.42 per cent dl-methionine, respectively (see the data under preferred conditions in Table VIII). On the whole, the recoveries of methionine from the amino acid test mixtures were less satisfactory with *Lactobacillus fermenti*, *Leuconostoc mesenteroides*, and *Lactobacillus arabinosus* under the other conditions shown in Table VIII.

TABLE VIII

Summary of Recovery Data for Methionine from Amino Acid Test Mixture*

	Amino acid test mixture No.		
	1	2	3
Lactobacillus fermenti; preferred conditions†			
	per cent	*per cent*	*per cent*
A.D.‡	2.4	2.7	2.6
Range§	96.9–105.0	95.8–103.8	98.2–103.3
Mean‖	101.4 ± 1.7 (13)	100.6 ± 1.7 (10)	100.6 ± 1.0 (6)
Lactobacillus fermenti; all other conditions¶			
A.D.	3.3	3.5	2.8
Range	95.0–116.2	98.1–117.5	92.0–119.0
Mean	103.3 ± 3.1 (13)	103.4 ± 3.6 (13)	102.5 ± 3.5 (9)
*Leuconostoc mesenteroides***			
A.D.	4.5	9.3	5.8
Range	89.5–113.0	83.6–118.6	86.0– 99.8
Mean	96.6 ± 4.0 (6)	99.3±12.8 (3)	93.3 ± 4.9 (3)
Lactobacillus arabinosus††			
A.D.	4.0	5.8	3.4
Range	98.3–108.2	103.9–111.9	103.4–117.0
Mean	104.4 ± 4.1 (3)	107.2 ± 3.1 (3)	108.1 ± 6.0 (3)

* The amino acid test mixtures were the same as those referred to in Tables I, II, and III.

† The basal medium (Medium C, Table I, Paper XXIV (10)). The inoculum was diluted with a volume of sterile saline 3 to 9 times that of the medium from which it was centrifuged. The incubation time was 2 days.

‡ Average mean deviation from the mean of the values at the five levels of the sample.

§ Range of all assay values.

‖ Mean assay value ± its mean deviation from the mean. The figures in parentheses denote the number of assays.

¶ The concentration of total amino acids in the basal medium varied from 1 to 2 times that referred to above (†), the incubation times varied from 1 to 3 days, and the dilution of inoculum suspension varied from 1:1 to 1:27. Seven sets of conditions were employed.

** The basal medium was the same as that given in Paper XVIII (Medium D, Table I) (9) except that the concentration of total amino acids was 1.33 times that given in the table. The incubation time was 5 days.

†† The conditions were the same as those given above (†) except that the incubation time was 3 days. The composition of the basal medium was the same as that reported by Shankman (3) except that the higher vitamin levels given in later papers from the authors' laboratory were employed and cysteine hydrochloride was substituted for cystine at about half the equivalent concentration stipulated by Shankman. High blank titration values were observed at higher concentrations of cysteine.

TABLE IX
Summary of Protein Assay and Recovery Data*

	Methionine in casein		Methionine in silk fibroin	
	Found	Recovered†	Found	Recovered†

Lactobacillus fermenti; preferred conditions

	per cent	per cent	per cent	per cent
A.D.	0.09	4.4	0.005	7.2
Range	2.87 – 3.13	88.7 – 103.5	0.128 – 0.140	95.2 – 107.8
Mean	3.01 ± 0.07 (7)	97.8 ± 4.0 (7)	0.136 ± 0.003 (6)	100.9 ± 3.7 (5)

Lactobacillus fermenti; all other conditions

A.D.	0.12	2.9	0.006	3.9
Range	2.83 – 3.19	96.6 – 105.4	0.126 – 0.153	62.5 – 107.0
Mean	2.96 ± 0.08 (10)	100.2 ± 2.6 (10)	0.139 ± 0.006 (10)	89.9 ± 8.4 (9)

Leuconostoc mesenteroides

A.D.	0.14	4.1	0.011	5.2
Range	2.69 – 3.06	88.4 – 99.4	0.103 – 0.134	84.2 – 123.2
Mean	2.84 ± 0.15 (3)	95.2 ± 4.5 (3)	0.115 ± 0.013 (3)	101.8 ± 14.3 (3)

Lactobacillus arabinosus

A.D.	0.11	3.4	0.005	2.1
Range	2.74 – 3.08	105.4 – 106.0	0.119 – 0.135	106.2 – 119.4
Mean	2.93 ± 0.13 (3)	105.7 ± 0.2 (3)	0.127 ± 0.005 (3)	112.5 ± 4.6 (3)

* The conditions described in the foot-notes to Table VIII were employed. The percentages of casein and silk fibroin designated as "found" refer to the moisture- and ash-free proteins.

† See foot-note 2 of the text.

The assay values for methionine in casein and in silk fibroin agreed closely and the recoveries[2] of methionine from the acid hydrolysates of these proteins did not deviate markedly from 100 per cent. The determination of

[2] Recoveries were calculated, as shown in Paper XXV (1), on the basis of the pure amino acid added to an aliquot of the protein hydrolysate. Deviations from 100 per cent calculated on this basis are approximately twice those calculated on the basis of the sum of the amino acid added and that found by assay to be present in the aliquot of the hydrolysate when the quantity of the amino acid derived from both sources is approximately the same. Recovery percentages calculated by the latter method have been reported by Stokes et al. (39) and they may have been calculated in this manner by other workers. The former procedure is preferred by the present authors, since it provides a more rigorous test of the accuracy of microbiological methods.

methionine with *Leuconostoc mesenteroides* and *Lactobacillus arabinosus*[8] was considered to be less dependable than that with *Lactobacillus fermenti*

TABLE X

Per Cent of Methionine in Casein[*] (*Literature Values*)

Hydrolysis procedure	Analytical method	Methionine[†]		Bibliographic reference No.
		Range	Average	
		per cent	*per cent*	
Concentrated HI; reflux 5–15 hrs. with and without N_2	Volatile iodide	2.6–3.5	3.2	(22–27)
Concentrated HI; reflux 6 hrs. with and without N_2	Homocysteine	2.9–3.1	3.1	(24, 28–30)
6–7 N HCl; reflux 6–10 hrs. at temperature up to 135°	Colorimetric	2.7–3.7[‡]	3.3	(31–34)
6 N HCl; reflux 7 hrs.	Periodide titration	2.8, 2.9	2.8	(35)
Concentrated HI; reflux 18 hrs.	Gravimetric (cuprous mercaptide of homocysteine)		3.1	(36)
7 N HCl; reflux 24 hrs.	H_2O_2-$HClO_4$ oxidation	2.8–2.9	2.8	(37)
	Differential oxidation		2.9	(38)
10% HCl; reflux 5–10 hrs. at 121°	Microbiological[§]		2.6	(39)

* In ten cases the source of the casein samples was not given. Five of the casein samples were Labco, four Harris, three Van Slyke, two Hammarsten, one Sheffield, and one Difco. All of the caseins were corrected for moisture or moisture and ash or calculated to 16 per cent nitrogen.

† From 0.06 to 1.4 per cent of methionine has been isolated from casein as the mercuric sulfate or acetate complex (16–21).

‡ The values, 4.7 and 4.9 per cent, given by Block and Bolling (31) have been omitted.

§ Methionine determined with *Streptococcus faecalis*.

because of the relatively high deviations from the mean of the recoveries from the amino acid test mixtures and the protein hydrolysates. It is not

[8] Since it has been reported (3) that there may be two strains of *Lactobacillus arabinosus* which have been employed for assay purposes, experiments were performed to determine whether or not cultures of this organism would respond similarly to methionine. The recoveries of methionine from an amino acid test mixture simulating the composition of casein averaged 102 (95 to 107) per cent with cultures of *L. arabinosus* 17-5 including those obtained from the American Type Culture Collection (one carried for a few months and one for 3 years on yeast-dextrose agar in the authors' laboratory, the Western Regional Research Laboratory (Albany, California) through the courtesy of Dr. J. C. Lewis, and the Agricultural and Mechanical College (College Station, Texas) through the courtesy of Dr. C. M. Lyman. It appears probable, therefore, that the four cultures were the same strain of *L. arabinosus*.

improbable, however, that assays of higher dependability could be made with these microorganisms provided conditions were established more nearly optimal than those employed.

Methionine in Casein[4]

The percentage of methionine determined with *Lactobacillus fermenti* under the best conditions was 3.01 (2.87 to 3.13, range of seven assays) per cent, corrected for the 6.21 per cent moisture and 0.55 per cent ash in this preparation as reported in Paper XVII (11). Other percentages of methionine (given in Table IX) found under other conditions were 2.96 (2.83 to 3.19, range of ten assays) per cent, determined with *Lactobacillus fermenti*, 2.87 (2.69 to 3.06, range of three- assays), determined with *Leuconostoc mesenteroides*, and 2.93 (2.74 to 3.08, range of three assays) determined with *Lactobacillus arabinosus*.

It seems probable that the true value for methionine in casein is 3.0 ± 0.1 per cent. This conclusion is strongly supported by the data given in Table X, since nearly the identical value has been reported by authors from ten laboratories who determined methionine by seven methods, differing in principle, in samples of casein prepared by the Hammarsten, the Van Slyke and Baker, and other procedures. Except for the low values obtained by isolation procedures, nearly all of the values for methionine given in Table X are not more than about 10 per cent lower or about 20 per cent higher than 3.0 per cent. It is of interest that methionine has been determined in proteins by more methods differing in principle than any other amino acid, that the percentages of methionine in casein obtained by different methods are in such close agreement, and that approximately the same percentage of methionine has been found in casein prepared from different milk samples by a variety of procedures. It would appear either that casein, irrespective of its source and method of preparation, contains nearly constant proportions of α- and β-caseins[5] or that α- and β-caseins contain nearly the same percentage of methionine.

Methionine in Silk Fibroin[4]

The content of methionine determined with *Lactobacillus fermenti* under the best conditions was 0.136 (0.128 to 0.140, range of six assays) per cent,

[4] It appears that casein and silk fibroin contain little, if any, $d(+)$-methionine since the methionine content of each protein determined with *Lactobacillus fermenti* (giving the sum of the $l(-)$- and the $d(+)$-methionine) was nearly the same as that determined with *Leuconostoc mesenteroides* or *Lactobacillus arabinosus* (giving only $l(-)$-methionine). It should be possible, however, to determine by differential assay with *Lactobacillus fermenti* and one of the other microorganisms the quantities of $l(-)$- and $d(+)$-methionine present in a mixture of these antipodes.

[5] The evidence for the non-homogeneity of casein has been reviewed by Warner (40).

'corrected for the 5.68 per cent moisture and 0.25 per cent ash reported in Paper XVII (11). Other values of methionine (given in Table IX) found under other conditions were 0.139 (0.126 to 0.149, range of eleven assays) per cent, determined with *Lactobacillus fermenti*, 0.115 (0.103 to 0.134, range of three assays) per cent, determined with *Leuconostoc mesenteroides*, and 0.127 (0.119 to 0.135, range in three assays) per cent, determined with *Lactobacillus arabinosus*. It appears that methionine may be assayed in silk fibroin under the described best conditions with an accuracy comparable to that attainable in the microbiological assay of amino acids in other proteins.

The methionine content of oven-dried (105°) silk fibroin obtained from Dr. W. H. Stein and Dr. Max Bergmann was found to be 0.15 per cent by Stokes *et al.* (39) who determined methionine microbiologically with *Streptococcus faecalis*. No other values have been found in the literature.[6]

SUMMARY

Microbiological procedures have been described for the determination of methionine in protein hydrolysates with the microorganisms *Lactobacillus fermenti* 36, *Leuconostoc mesenteroides* P-60, and *Lactobacillus arabinosus* 17-5. The methionine in casein and silk fibroin, respectively, was found to be 3.01 and 0.136 per cent under the conditions considered to be most dependable (assay with *Lactobacillus fermenti* inoculum diluted with a volume of sterile saline 3 to 9 times that of the medium from which it was centrifuged and incubated for 2 days at 35° in a basal medium (Medium C, Table I) of a composition given in Paper XXIV (10)).

It is considered that the probable true values for methionine in these proteins are 3.0 ± 0.1 (casein) per cent and 0.14 ± 0.01 (silk fibroin) per cent, although it is recognized that the extent to which methionine is destroyed during hydrolysis must be measured before the true values can be determined. The nearly identical value for methionine in casein has been reported by authors from ten laboratories who determined methionine by seven methods, differing in principle, in samples of casein prepared by several different procedures.

The growth of *Lactobacillus fermenti* was stimulated equally by the $d(+)$ and the $l(-)$ antipodes of methionine. This response appears to be a unique property of microorganisms not hitherto recorded in the literature.

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[6] Tomiyama (41) has reported 0.42 per cent of methionine in silkworm pupa (moisture- and ash-free). The value, 2.59 per cent, given by Cohn and Edsall (42), is incorrect, since it was reported for fibrin by Baernstein (22), the author quoted.

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INVESTIGATIONS OF AMINO ACIDS, PEPTIDES, AND PROTEINS

XXVII. THE DETERMINATION OF THREONINE IN PROTEIN HYDROLYSATES WITH LACTOBACILLUS FERMENTI 36[*]

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It has been reported that threonine[1] is essential or is accessory for the growth of *Lactobacillus arabinosus* 17-5 (6–11), *Leuconostoc mesenteroides* P-60 (12), *Lactobacillus delbrückii* (5), *Streptococcus faecalis* (*Streptococcus lactis* R) (4), and *Lactobacillus fermenti* 36 (13). The first microbiological determination of threonine was that of Shankman *et al.* (8) who found by assay with *L. arabinosus* 94 per cent of the threonine present in a mixture of eleven amino acids. The percentage of threonine in corn, wheat, rye, and barley and the alcohol fermentation by-products of these cereal grains was determined with *L. arabinosus* by Baumgarten *et al.* (11) who reported recoveries of threonine, added to samples prior to hydrolysis, ranging from 95 to 105 per cent. Threonine in six purified proteins and fourteen natural products has been determined with *S. faecalis* by Stokes *et al.* (4) who showed that *dl*-allothreonine, the unnatural isomer of threonine,[2] α-bromo-β-

[1] Threonine is an accessory but not an essential substance for *Lactobacillus casei* according to Hutchings and Peterson (2). The statement of Stokes and Gunness (3) is not entirely clear on this point. Stokes *et al.* (4) have stated recently that pyridoxamine or pyridoxal can substitute for threonine in the nutrition of the lactobacilli which, according to an earlier report by Stokes and Gunness (5), included *L. casei*, *L. arabinosus*, and *L. delbrückii* but not *S. lactis* R. These conclusions were drawn from experiments in which the basal medium of Hutchings and Peterson (2) was employed. It has been found in the present experiments that the requirement of *L. fermenti* for threonine was not eliminated either by pyridoxamine and pyridoxal when this organism was grown on the authors' synthetic medium. The rate of synthesis of threonine was accelerated under some conditions in the presence of these substances but no other effects were noted. The pyridoxamine and pyridoxal employed in these experiments were obtained from Merck and Company through the courtesy of Dr. J. L. Stokes.

[2] Stokes *et al.* (4) designated the natural isomer of threonine as *l*(−)-threonine and the unnatural isomer as *d*(+)-threonine, the letters *l* and *d* being employed to

methoxybutyric acid, and four derivatives of threonine were inactive in promoting the growth of this organism. The assays at different levels of sample, the successive assays on the same sample, and the recoveries[3] of threonine added prior to hydrolysis of two purified proteins and three natural foods reported by Stokes et al. were in reasonably close agreement.

The determination of threonine in protein hydrolysates with *Lactobacillus fermenti* 36 is described in this paper.

<p align="center">EXPERIMENTAL</p>

A basal medium essentially of the composition given (Medium C, Table I) in Paper XXIV (13) and the assay technique described in Paper XXVI (1) were employed. 3 drops of 0.8 per cent dibromothymolsulfonephthalein (bromothymol blue) indicator solution (50 per cent ethanol) were required for the 3 ml. of experimental solution in each test-tube to give a satisfactory end-point in the presence of the marked discoloration produced by caramelization of the amino acids present at relatively high concentration in the basal medium. The standard was run at fifteen levels up to 84 γ of *dl*-threonine, the amino acid test mixtures and protein hydrolysates were run at five levels, and six tubes were employed at each level of standard and sample. The total volume of solution in each tube (4 inch) was 3 ml.

The activity of *dl*-allothreonine and unnatural *l*(+)-threonine was not tested but it was considered probable from the data reported by Stokes et al. (4) for *Streptococcus faecalis* that these substances would not promote the growth of *Lactobacillus fermenti*. Identical results were obtained in assays with *dl*-threonine purchased from Merck and Company and that prepared in this laboratory by William Blackwood.

The casein, the silk fibroin, and the hydrolysis procedure were the same as those described in Paper XVII (14). The experimental data are given in Tables I to VIII. The basal medium given in Paper XXIV (Medium C, Table I) (13), except that the concentration of total amino acids was

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denote the relative configuration of the α-carbon atom in harmony with the common practice of naming the other amino acids. It has been the custom, previously, to designate natural threonine as $d(-)$-threonine because both asymmetric carbon atoms in natural threonine and D-threose have the same relative configuration.

[3] Stokes et al. (4) have calculated recoveries from the equation $C(A + B) \times 100$ = per cent recovery, where A is the quantity of amino acid found by assay to be present in the aliquot of the hydrolysate taken, B is the quantity of amino acid added, and C is the amino acid found in the mixture of A and B. Recoveries are calculated by the present authors from the equation $(C - A)/B \times 100$ = per cent recovery, where the letters A, B, and C have the same meaning. The recovery of threonine calculated by Stokes et al. from the first equation was 102 per cent and the recovery would be 105 per cent if calculated from the second equation. It appears that true recovery may be calculated only by the latter procedure.

TABLE I

Assay of Threonine in Amino Acid Test Mixture 1*

The test mixture contained 2.50 per cent $d(-)$-threonine (added as dl-threonine).

Amino acid mixture per tube	$d(-)$-Threonine present per tube	Titration volume†		$d(-)$-Threonine found per tube	$d(-)$-Threonine recovered
		Range	Average		
γ	γ	*ml.*	*ml.*	γ	*per cent*
281.3	7.02	3.15–3.37	3.23	7.00	99.7
562.6	14.04	3.93–4.23	4.06	13.70	97.6
843.9	21.06	5.09–5.52	5.32	21.30	101.1
1125.2	28.07	6.20–6.80	6.53	28.90	103.0
1406.5	35.09	7.29–8.01	7.54	36.10	102.9
Average..					100.9

* The composition of the test mixture simulating that of casein was the same as that given in Paper XXV (15).

† The solution in each of six replicate tubes at each level of sample was titrated with 0.0310 N NaOH.

TABLE II

Assay of Threonine in Amino Acid Test Mixture 2*

The test mixture contained 1.67 per cent $d(-)$-threonine (added as dl-threonine).

Amino acid mixture per tube	$d(-)$-Threonine present per tube	Titration volume†	$d(-)$-Threonine found per tube	$d(-)$-Threonine recovered
γ	γ	*ml.*	γ	*per cent*
360	6.00	7.69	5.62	93.7
720	12.00	11.71	12.00	100.0
1080	18.00	16.60	17.82	99.0
1440	24.00	19.22	25.25	105.0
1800	30.00	20.91	29.50	98.3
Average..				99.2

* The composition of the text mixture was essentially the same as that given in Paper XXV (15).

† The solutions of six replicate tubes at each level of sample were mixed and titrated with 0.0623 N NaOH.

2.5 times that stated, and 2 day incubation at 35° with *Lactobacillus fermenti* were employed in all assays reported in these tables.[4]

[4] It was shown in Figs. 14 and 27, Paper XXIV (13), that 1 times the concentration of total amino acids (equivalent to amino acid Level 8 in Fig. 14) was the lowest level of total amino acids in the basal medium and that a 2 day incubation period was the maximum time which probably would be satisfactory for the assay of threonine under the other conditions described. It has been reported in the present paper that 2.5 times the concentration of total amino acids (equivalent to amino acid Level 20 in Fig. 14) and 2 day incubation time were the most satisfactory assay conditions.

DISCUSSION

It has been found that threonine could be determined most accurately by incubating a *Lactobacillus fermenti* inoculum (diluted with a volume of sterile saline solution from 3 to 9 times that of the medium from which it

TABLE III

Assay of Threonine in Amino Acid Test Mixture 8*

The test mixture contained 0.98 per cent $d(-)$-threonine (added as dl-threonine).

Amino acid mixture per tube	$d(-)$-Threonine present per tube	Titration volume†	$d(-)$-Threonine found per tube	$d(-)$-Threonine recovered
γ	γ	ml.	γ	per cent
626.6	6.17	5.52	5.80	94.0
1253.2	12.34	7.40	12.05	97.6
1879.8	18.52	9.18	18.90	102.1
2506.4	24.69	10.90	24.10	97.6
3133.0	30.86	12.20	30.10 ·	97.5
Average..				97.8

* The composition of the test mixture simulating that of silk fibroin was the same as that given in Paper XXV (15).

† The solutions of six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

TABLE IV

Assay of Threonine in Casein Hydrolysate

Hydrolyzed casein (moisture- and ash-free) per tube	Titration volume*	$d(-)$-Threonine found per tube	$d(-)$-Threonine in casein
γ	ml.	γ	per cent
149.2	5.62	6.10	4.09
298.4	7.52	12.50	4.19
447.6	9.60	20.85	4.66
596.7	11.02	24.50	4.11
745.9	12.60	31.25	4.19
Average..			4.25

* The solutions of six replicate tubes at each level of sample were mixed and titrated with 0.105 N NaOH.

was centrifuged) for 2 days at 35° in a basal medium (Medium C, Table I (13)) except that the concentration of total amino acids was 2.5 times that stated. Under these conditions, the average mean deviation from the mean values at the five levels of each sample was about 3.5 per cent for the three amino acid test mixtures described in Tables I to III, which con-

tained 2.50, 1.67, and 0.98 per cent $d(-)$-threonine, respectively. The assay values for threonine in casein and silk fibroin and the recoveries of threonine from the acid hydrolysates of these proteins were reasonably

TABLE V

Recovery of Threonine Added to Casein Hydrolysate*

Hydrolyzed casein (moisture- and ash-free) per tube	$d(-)$-Threonine			
	In casein per tube†	Added per tube	Found per tube	Recovered
γ	γ	γ	γ	*per cent*
74.6	3.17	3.00	6.00	94.3
149.2	6.34	6.00	12.44	101.7
223.8	9.51	9.00	19.00	105.4
298.4	12.68	12.00	25.30	105.2
373.0	15.85	15.00	31.60	105.0
Average...				102.3

* The sample was prepared to contain 15.0 γ of $d(-)$-threonine (added as *dl*-threonine) per ml. and 373.0 γ of casein (moisture- and ash-free) per ml. Volumes from 0.20 to 1.00 ml. were taken for the assays.

† Estimated on the basis of the 4.25 per cent of threonine in casein given in Table IV.

TABLE VI

Assay of Threonine in Silk Fibroin Hydrolysate

Hydrolyzed silk fibroin (moisture- and ash-free) per tube	Titration volume*		$d(-)$-Threonine found per tube	$d(-)$-Threonine in silk fibroin
	Range	Average		
γ	*ml.*	*ml.*	γ	*per cent*
451.5	2.94–3.11	3.03	5.50	1.22
903.1	3.69–3.78	3.73	11.60	1.29
1354.6	4.32–4.61	4.42	15.90	1.17
1806.2	5.07–5.43	5.27	21.00	1.16
2257.7	5.61–6.29	5.91	24.84	1.10
Average...				1.19

* The solution in each of six replicate tubes at each level of sample was titrated with 0.0310 N NaOH.

satisfactory, although, on the whole, they were somewhat less precise and accurate than the methionine data reported in Paper XXVI (1).

The average of from nine to fourteen threonine values found under other assay conditions[5] was as follows: 107 (98.8 to 120) per cent, amino acid Test

[5] Concentration of total amino acids in the basal media and incubation times (given in parentheses) were as follows: 1 (2), 1.5 (2), 2 (2), and 2.5 (1).

Mixture 1; 110 (98.7 to 151) per cent, amino acid Test Mixture 2; 93.0 (84.8 to 101) per cent, amino acid Test Mixture 3; 4.5 (4.09 to 4.97) per cent, casein; 1.20 (0.93 to 1.40) per cent, silk fibroin; 98.4 (67 to 117) per cent, casein recovery; and 103 (87 to 131) per cent, silk fibroin recovery. These data and assay conditions were considered to be unsatisfactory.

The determination of threonine was investigated with *Leuconostoc mesenteroides* P-60 and a basal medium (Medium D, Table I (12)) except that the concentration of total amino acids was varied from 1 to 3.3 times that stated. Although there was no synthesis of threonine in 5 days, the standard curves were too irregular to yield dependable assays. It had been found previously (12) that threonine appeared to be essential for the

TABLE VII

Recovery of Threonine Added to Silk Fibroin Hydrolysate*

Hydrolyzed silk fibroin (moisture- and ash-free) per tube	$d(-)$-Threonine			
	In silk fibroin per tube†	Added per tube	Found per tube	Recovered
γ	γ	γ	γ	*per cent*
225.8	2.69	3.00	5.70	100.3
451.5	5.37	6.00	12.30	115.5
677.3	8.06	9.00	16.50	93.8
903.1	10.75	12.00	21.70	91.3
1128.8	13.43	15.00	26.30	85.8
Average...				97.3

* The sample was prepared to contain 15.0 γ of $d(-)$-threonine (added as *dl*-threonine) and 1128.8 γ of silk fibroin (moisture- and ash-free) per ml. Volumes from 0.20 to 1.00 ml. were taken for the assays.

† Estimated on the basis of the 1.19 per cent of $d(-)$-threonine in silk fibroin given in Table VI.

growth of *Leuconostoc mesenteroides* P-60 but it was considered doubtful that threonine could be determined quantitatively under the conditions described.

The earlier report of Shankman *et al.* (8) that about 94 per cent of threonine in a mixture of eleven amino acids was recovered suggested that threonine in protein hydrolysates could be determined quantitatively with *Lactobacillus arabinosus* 17-5. This assumption was invalidated, however, by the observation that the recoveries of threonine added to amino acid Test Mixture 1 ranged from 120 to 230 per cent from assays with the four cultures of *Lactobacillus arabinosus* 17-5 (1) and the basal medium (7) described previously.

Threonine in Casein

The threonine determined with *Lactobacillus fermenti* under the best conditions was 4.27 (4.11 to 4.51, range of five assays) per cent, corrected for the 6.21 per cent moisture and 0.55 per cent ash reported in Paper XVII (14). If it is assumed that there is no appreciable destruction of

TABLE VIII

Summary of Threonine Assay and Recovery Data*

Amino acid test mixture No.; recovery†			Casein		Silk fibroin	
1	2	3	Found	Recovered	Found	Recovered
per cent	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*	*per cent*
108.0	101.0	98.6	4.11	100.3	1.36	97.5
99.2	102.0	95.4	4.25	98.6	1.19	92.5
100.9	97.9	93.5	4.23	92.6	1.19	114.0
104.8	104.4	91.5	4.25	96.7	1.21	106.1
97.3	102.3	97.8	4.51	102.3	1.26	93.0
99.4	97.7	97.0		96.0	1.21	
99.0	101.7					
93.6	95.0					
101.0	94.6					
96.6	101.2					
100.8	99.2					
99.4						
98.7						
99.0						
100.6	100.0	95.5	4.27	97.8	1.24	100.6

* The basal medium was the same as that given in Paper XXIV (Medium C, Table I) (13) except that the concentration of total amino acids was 2.5 times greater. The incubation time was 2 days. The average mean deviations from the mean of the threonine values found at the different levels of samples were (in per cent) 3.5 (1.6 to 7.0), amino acid Test Mixture 1; 4.0 (2.0 to 6.7), amino acid Test Mixture 2; 3.2 (1.8 to 5 0), amino acid Test Mixture 3; 5.3 (1.2 to 8.2), casein assay; 2.9 (1.1 to 6.7), casein recovery; 3.8 (1.0 to 9.0), silk fibroin assay; and 4.5 (3.8 to 6.7), silk fibroin recovery.

† The amino acid test mixtures were the same as those referred to in Tables I, II, and III.

threonine during acid hydrolysis, it seems probable that the true value for threonine in casein is 4.3 ± 0.2 per cent. Four closely agreeing values (bold-faced type in Table IX) were obtained in four laboratories by the periodic acid-iodometric titration procedure and by microbiological assay with *Streptococcus faecalis*.

TABLE IX

*Percentage of Threonine in Casein (Literature Values)**

Casein sample	Hydrolysis procedure	Analytical method†	Threonine	Bibliographic reference No.
			per cent	
Source not given	20% HCl overnight; reflux	A	3.5‡	(18)
" " "	20% H₂SO₄ 16 hrs. "	B	3.3§	(19)
" " "	6 N HCl overnight "	C	4.6§	(20)
Hammarsten	20% " 24 hrs.; reflux	A	3.3‖	(21)
Source not given	20% " 24 " "	D	3.5¶	(22)
Commercial	HCl	"	2.9**	(23, 24)
Source not given	25% H₂SO₄	A	3.5††	(25)
Commercial	20% HCl 24 hrs.; reflux	D	4.0‡‡	(26)
T. B. Osborne	3 N " 26 " "	"	4.3§§	(27)
S. M. A., vitamin-free	20% " 13 " "	"	3.5§	(28)
Source not given	No data	"	4.6‖‖	(29)
S. M. A.	10% HCl 10 hrs.; 121°	E	4.2¶¶	(4)

* A 4.5 gm. yield (equivalent to 0.08 per cent) of purified threonine was isolated from fibroin by McCoy *et al.* (16) in 1935 but it appears that there are no data on the percentage of threonine isolated from casein.

† (A) lead tetraacetate oxidation to acetaldehyde and colorimetric determination of the acetaldehyde-*p*-hydroxydiphenyl complex; (B) bromine oxidation to a diketone and colorimetric determination in the presence of ferric ions of the diketoxime formed from the diketone and hydroxylamine; (C) periodic acid oxidation to acetaldehyde and colorimetric determination of the acetaldehyde-*p*-hydroxydiphenyl complex; (D) periodic acid oxidation to acetaldehyde and iodometric titration of the bisulfite-bound acetaldehyde; (E) microbiological assay with *Streptococcus faecalis* (*Streptococcus lactis* R).

‡ For casein containing 14.7 per cent nitrogen according to Block and Bolling (17).

§ No other data available.

‖ For casein (containing 15.08 per cent nitrogen, 0.82 per cent phosphorus, 0.63 per cent sulfur, and 1.10 per cent ash) dried at 105°. The value listed was the average of 2.8 and 3.7 per cent found in two determinations.

¶ Average of nine values (3.43 to 3.68 per cent) for casein uncorrected for moisture and ash. The nitrogen content of the casein was not given. Recoveries of threonine from four mixtures containing from three to five amino acids ranged from 96 to 98 per cent. Recoveries from casein hydrolysates averaged 96 (93 to 97) per cent.

** Value for British Drug Houses casein (containing 13.4 per cent nitrogen) calculated to 16.0 per cent nitrogen. The value was considered to be 30 to 35 per cent low, since the recovery of threonine from a mixture containing fifteen amino acids was 70 per cent.

†† Value for casein R. Value, 3.2 per cent, found for casein R by the method of Shinn and Nicolet (22). The value, 1.0 per cent, was found by the method of Block and Bolling (18) and the value, 0.7 per cent, was found by the method of Shinn and Nicolet (22) for casein A-165 hydrolyzed by heating it for 8 hours at 165° with 10 per cent H₂SO₄. Moisture, ash, and nitrogen were not given for either casein sample.

‡‡ Value for fat-free commercial casein (containing 14.00 per cent nitrogen) calculated to 15.80 per cent nitrogen.

TABLE IX—*Concluded*

§§ Value for sample of T. B. Osborne casein (containing 14.75 per cent nitrogen on moisture- and ash-free basis) furnished by Dr. H. B. Lewis. The average value, 4.3 per cent, was calculated from about seven determinations ranging from 4.10 to 4.46 per cent. 'The average value was 4.7 per cent for the casein corrected to 16.0 per cent nitrogen. Recoveries of threonine added to casein hydrolysates ranged from 96 to 99 per cent.

|||| For casein (containing 15.28 per cent nitrogen on ash- and moisture-free basis) prepared by isoelectric precipitation.

¶¶ For casein (dried at 105°) assayed with *Streptococcus faecalis.*

Threonine in Silk Fibroin

The threonine determined with *Lactobacillus. fermenti* under the best conditions was 1.24 (1.19 to 1.36, range of six assays) per cent, corrected for the 5.68 per cent moisture and 0.25 per cent ash reported in Paper XVII (14). The following values for threonine in silk fibroin have been reported previously: 0.62 per cent, found by Martin and Synge (24) for silk fibroin containing 18.3 per cent nitrogen; 1.54 (1.43 to 1.66, range of five determinations) per cent, found by Nicolet and Saidel (30) for silk fibroin (dried in a vacuum desiccator) obtained from Dr. M. Harris; and 1.36 per cent found by Stokes *et al.* (4) for silk fibroin (dried at 105°) obtained from Dr. W. H. Stein and Dr. M. Bergmann. The first two values were determined by the periodic acid-iodometric titration procedure of Shinn and Nicolet (22). The last value was determined by microbiological assay with *Streptococcus faecalis.*

SUMMARY

A microbiological procedure has been described for the determination of threonine in protein hydrolysates with *Lactobacillus fermenti* 36. The percentages corrected for moisture and ash of threonine in casein and silk fibroin, respectively, were found to be 4.3 and 1.2 under the conditions considered to be most dependable.

It is considered probable that the true values for threonine in these proteins are 4.3 ± 0.2 (casein) and 1.2 ± 0.2 (silk fibroin) per cent, although it is recognized that the extent to which threonine is destroyed during hydrolysis must be measured before the true values can be determined. Percentages of threonine in casein and silk fibroin which are in fair agreement with the authors' values have been found in other .laboratories by the periodic acid-iodometric titration procedure and microbiological assay with *Streptococcus faecalis.*

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SOME RELATIONSHIPS BETWEEN THE AMINO ACID CONTENTS OF PROTEINS AND THEIR NUTRITIVE VALUES FOR THE RAT

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It is obvious that the nutritive value of a protein or mixture of proteins for any biological function or combination of functions is limited by the relative proportions of the essential amino acids contained in it; *i.e.*, those amino acids that cannot be synthesized by the animal at a sufficiently rapid rate from any substances present in the usual diets. But it is not so clear that the amino acid make-up of a protein is the only considerable factor limiting its utilization within the animal body.

The experiments of Sherman and Woods (1) on the determination of cystine in proteins by means of feeding experiments with growing rats afford an illustration of a close relationship between amino acid content and the growth-promoting value of protein, though perhaps not sufficiently close to justify using a rat growth technique to check the accuracy of a chemical assay method for an amino acid, as Grau and Almquist (2) have done in their study of the methionine content of various feed proteins. With much the same conviction, Munks, Robinson, Beach, and Williams (3) have assessed the amino acid requirements of the laying hen for the production of one egg as being equal to the amino acids contained in one egg. Undoubtedly, this is a good basis on which to assess the net amino acid requirements, but it may afford quite uncertain information of the amino acid intakes from different protein combinations needed to cover these requirements.

Comparison of Chemical and Biological Methods of Assaying Protein Quality

The study of the relationship between the amino acid constitution of proteins and their value in animal growth may be pursued further by comparing the amino acid contents of certain food products, as determined by modern methods, with the results of rat feeding experiments designed to detect the amino acids limiting their value in the nutrition of growth. For this purpose, use has been made of the amino acid analyses of food products re-

cently assembled by Block and Bolling (4),[1] and later discussed by them (6), from the standpoint of nutritional applications. These values have been revised and supplemented to some extent by more recent analyses. The relative amino acid deficiencies of food proteins in the nutrition of the growing rat can be revealed clearly only by a comparison of the proportions of the essential amino acids present in the proteins with the proportions existing among the amino acid requirements of the rat for growth. These at present are unknown. However, in the proteins of whole egg we have an amino acid mixture that is very highly digestible and almost perfectly utilizable in rodent metabolism, being better than milk proteins in this respect. This was first shown by Mitchell and Carman (7) for the growing rat and was later confirmed by Sumner (8) for both growing and mature rats. For the adult human subject, also, whole egg proteins seem to be better utilized than whole milk proteins (9, 10).

In Table I are given the percentage deviations of the contents of different food proteins in the essential amino acids, in Rose's sense of the term, and also in tyrosine and cystine, from the contents of the corresponding amino acids found in whole egg protein.[2] The first column of the values represents the results of a recent analysis of a dried preparation of whole egg performed by one of the authors (R. J. B.).[3] The methods used in this analysis will be given later. Each value in the other columns expresses the percentage deviation in the amino acid content of a specified protein mixture (standardized to a nitrogen content of 16 per cent) from that of the proteins of dried whole egg. For example, beef muscle proteins, according to available analyses, contain 46 per cent less cystine than whole egg proteins, and 12 per cent more lysine. The amino acid limiting the nutritional value for maintenance and growth of the laboratory rat for any particular food protein would be that amino acid present in the least amount with reference to

[1] The recent communication of Vickery and Clarke (5), criticizing the method used by Block and Bolling of computing the amino acid content of proteins to a uniform protein content of 16 per cent nitrogen, expresses the viewpoint of the protein chemist concerned solely with problems of protein structure. The viewpoint of the protein nutritionist, however, is entirely different, because the utilization of dietary proteins by animals can be studied only by the nitrogen balance sheet method at the present time. Hence, an amino acid analysis of a protein is most useful in protein nutrition as a chemical description of the nitrogen contained in it. From this standpoint, it is entirely immaterial whether the protein contains 15 or 18 per cent of nitrogen; in fact, for the most exact appraisal of a protein in nutrition, such differences in nitrogen content should be disregarded by computing amino acid contents on a conventional basis of 16 per cent of nitrogen.

[2] A somewhat similar use of the amino acid content of whole egg proteins has been made by Stare, Hegsted, and McKibbin (11).

[3] Dried and solvent-extracted at low temperatures by the VioBin Corporation, Monticello, Illinois, through the courtesy of Mr. Ezra Levin.

TABLE I

Percentage Deviations of Amino Acid Contents of Food Proteins from Amino Acid Contents of Proteins of Whole Egg Taken As Standard

Amino acid	Whole egg protein (per cent)	Percentage deviations from corresponding values for whole egg proteins										
		Egg albumin	Cow's milk	Casein	Lactalbumin	Human milk	Blood serum	Hemoglobin	Beef muscle	Heart	Kidney	Liver
Arginine	6.4	−11	−33	−34	−39	−33	−5	−45	+20	+16	−2	+3
Histidine	2.1	+14	+24	+43	0	+33	+48	+262	+38	+29	+29	+48
Lysine	7.2	−31	+4	+10	+33	0	+33	+25	+12	+3	−24	−7
Tyrosine	4.5	−7	+18	+53	−2	+16	+20	−47	−24	−2	+7	+2
Phenylalanine	6.3	+2	−10	−11	−14	−11	−14	+22	−22	−19	−13	−3
Tryptophane	1.5	−7	+7	−20	+67	+27	+13	0	−13	−7	+13	−7
Cystine	2.4	+21	−58	−87	+71	+42	+54	−25 to −83	−46	−50	−37	−42
Methionine	4.1	+34	−17	−15	−34	−46	−54	−56 " −88	−20	−22	−34	−22
Cystine + methionine	6.5	+29	−32	−42	+5	−14	−14		−29	−32	−35	−29
Threonine	4.9	−22	−8	−16	+10	−6	+29	+27	−6	−4	−6	−2
Leucine	9.2	+2	+23	+8	+13	+7	+96	+68	−16	−9	−13	−9
Isoleucine	8.0	−11	+6	−19	−20	−6	−62	−79 to −99	−21	−35	−30	−30
Valine	7.3	0	+15	−8	−12	+21	−18	+22	−21	−14	−27	−15
Indicated limiting amino acid		Lysine	Cystine + methionine	Cystine + methionine	Methionine	Methionine	Isoleucine	Isoleucine	Cystine + methionine	Isoleucine	Cystine + methionine	Isoleucine

TABLE I—Continued

Amino acid	Whole egg protein	Percentage deviations from corresponding values for whole egg proteins							
		Cotton seed	Flax seed	Sesame seed	Sunflower seed	Peanut	Soy bean	Pea	Alfalfa
	per cent								
Arginine	6.4	+16	+31	+44	+28	+55	+11	+39	−33
Histidine	2.1	+24	−29	−28	−19	0	+10	−43	0
Lysine	7.2	−63	−65	−61	−47	−58	−19	−31	−32
Tyrosine	4.5	−29	+13	−4	−42	−2	−9		+27
Phenylalanine	6.3	+8	−11	+32	−14	−14	−10	−24	−29
Tryptophane	1.5	−13	0	+27	−13	−33	−20	−53	+7
Cystine	2.4	−17	−21	−46	−46	−33	−21	−50	−33
Methionine	4.1	−49	−44	−24	−17	−51 to −76	−51	−76	−44
Cystine + methionine	6.5	−37	−35	−32	−28	−45 " −60	−40	−66	−40
Threonine	4.9	−39	+4	−27	−18	−69	−18	−20	−33
Leucine	9.2	−46	−24	−18	−33	−24	−28	−30	−28
Isoleucine	8.0	−58	−50	−40	−35	−62	−41	−49	−55
Valine	7.3	−49	−4	−30	−29	+10	−42	−45	−40
Indicated limiting amino acid		Lysine	Lysine	Lysine	Lysine	Methionine	Methionine	Methionine	Isoleucine

TABLE I—*Concluded*

Amino acid	Whole egg protein	Percentage deviations from corresponding values for whole egg proteins								
		Gelatin	Whole wheat	Wheat germ	White flour	Whole corn	Corn germ	White rice	Rolled oats	Average yeast
	per cent									
Arginine	6 4	+36	−34	−6	−39	−25	+27	+13	−6	−33
Histidine	2 1	−57	0	+19	+5	−5	+38	−29	+5	+33
Lysine	7 2	−19	−63	−24	−72	−72	−19	−56	−54	+4
Tyrosine	4 5	−84	−2	−16	−16	+22	+49	+24	+2	−20
Phenylalanine	6 3	−67	−10	−33	−13	−21	−13	+6	+5	−35
Tryptophane	1 5	−100	−20	−33	−33	−47	−13	−13	−20	−13
Cystine	2 4	−96	−25	−67	−21	−37	−25	−42	−25	−58
Methionine	4 1	−80	−39	−51	−63	−24	−61	−17	−41	−54
Cystine + methionine	6 5	−86	−34	−57	−48	−29	−48	−26	−35	−55
Threonine	4 9	−59	−33	−22	−45	−24	−4	−16	−29	+12
Leucine	9 2	−66	−26	−27	−18	+139	+41	−2	−10	−20
Isoleucine	8 0	−79	−55	−62	−54	−50	−50	−34	−30	−26
Valine	7 3	−62	−38	−44	−42	−32	−18	−14	−14	−32
Indicated limiting amino acid		Trypto-phane	Ly-sine	Isoleucine	Ly-sine	Ly-sine	Methionine	Ly-sine	Ly-sine	Cystine + methionine

whole egg proteins; *i. e.*, that amino acid with the greatest percentage deficit in Table I. Thus, the limiting amino acid in pea proteins is methionine (−76), that in blood serum proteins isoleucine (−62), and in wheat proteins lysine (−63).

The "indicated limiting amino acids" listed at the bottom of Table I are taken to be those essential amino acids in greatest percentage deficit. In such deductions, the arginine percentages are disregarded, since the growing rat can, to some unknown extent, supplement a dietary deficiency in this amino acid by its limited capacity to synthesize it. In view of the known relationship of cystine and methionine in metabolism, whereby methionine is convertible into cystine but the reverse reaction does not occur, the limiting factor between these two was assumed to be methionine, or methionine plus cystine, whichever percentage deficit is the greater. The latter designation means that the protein in question is supplemented fully by methionine and to some extent by cystine also. The same relationship exists between tyrosine and phenylalanine, but the necessity of distinguishing between these amino acids in this connection has not arisen in the construction of Table I.

The extent to which food proteins will supplement each other in a diet or ration will depend upon the identity or non-identity of their limiting amino acids, and, if they are not identical, upon the relative prominence of a common deficiency in some other essential amino acid. Thus, whole milk proteins should obviously supplement rice proteins because the limiting amino acid in the one case is cystine plus methionine and in the other case lysine, but the extent of supplementation would presumably be slight because rice proteins are also rather seriously deficient in the sulfur-containing amino acids.

Such uses of the values listed in Table I should be tempered by the fact that several methods of amino acid analysis are still quite imperfect.

The main purpose of presenting the data summarized in Table I is to compare the amino acid contents of food proteins with reference to the contents in whole egg proteins, with the results of rat feeding experiments designed to detect the limiting amino acids in the same food proteins. The largest percentage deficits in essential amino acids for the various food proteins considered in Table I are in harmony with the following conclusions as to the amino acids limiting the growth-promoting values of the proteins for the rat.

The proteins of whole milk (12), beef (13), soy beans (13–15), the peanut (16, 17), yeast (18), the pea (19), and casein (20, 21) are deficient in cystine or methionine or both. On the other hand, lactalbumin is definitely not deficient in cystine (21).

The proteins of wheat (13), oats (13), rye (22), corn (13, 23), rice (24), and

cottonseed (25) are deficient in lysine. The supplementation of corn and oats with lysine results in distinct, though slight, increase in growth-promoting power (13, 23) because of the interposition of secondary deficiencies, identified as tryptophane for corn proteins, but unidentified in the case of oat proteins. The data of Table I suggest that the second deficiency in oat proteins may be methionine.

Light and Frey (26) present evidence that white flour proteins are deficient in valine as well as lysine, a conclusion that finds some support in the values given in Table I for this food product, but the methionine and isoleucine deficits seem even more severe.

The proteins of blood plasma are primarily deficient in isoleucine (27). Hemoglobin, which is 79 per cent (or more) deficient in isoleucine, seems to be improved as a source of protein for the growing rat by a supplement of this amino acid (28).

Hegsted, Hay, and Stare (29) in their recent study of the nutritive value of human plasma fractions, employing the *ad libitum* feeding technique with young rats, found that serum albumin at a 20 per cent dietary level barely supported maintenance of body weight. The addition of isoleucine to the diet definitely improved the growth-promoting power of the ration, while the further addition of tryptophane brought about a marked improvement. Tryptophane alone had no appreciable effect. The values given in Table II, similar in significance to those in Table I, show that the greatest percentage deficits of serum albumin are 80 for tryptophane and 75 for isoleucine. These deficits in amino acid composition agree reasonably well with the biological tests reported by the Harvard group.

However, the amino acid constitution of proteins, as it is presented in Tables I and II, is not always in good agreement with reported biological tests with growing rats. Thus, the data of Table I indicate that soy bean proteins are deficient in methionine, although they are also supplemented by cystine (13–15). For alfalfa proteins, the greatest percentage deficit is that for isoleucine, although the biological evidence seems clear that they are deficient in cystine and methionine (30, 31). Marais and Smuts (32, 33) have reported evidence that linseed meal and sesame seed meal are improved in growth-promoting value by supplementation with cystine rather than with lysine, as the chemical data indicate. The fact that these workers use rats of much greater initial weight than usual may partially explain this discrepancy, a comment that is prompted by the fact that some of the results obtained with the technique of these workers do not harmonize with results secured in other laboratories, for example the failure to demonstrate a methionine deficiency in peanut meal (33) and a lysine deficiency in oats (32).

The computations in Table I may be used to compare the amino acid

compositions of food proteins expressed as percentage deviations from the
amino acid contents of the proteins of whole egg, with their biological values
for the growing rat, as determined by the nitrogen metabolism method,
developed by Mitchell (34, 7).[4]   One might expect that the proteins least
deficient in any limiting essential amino acid would possess a higher bio-
logical value than one more deficient, and that in general it would be pos-
sible to arrange food proteins in the order of decreasing biological efficiency
by placing at the head of the list the protein whose limiting amino acid is
least deficient, as compared with a nearly perfect mixture of amino acids
such as is found in whole egg proteins.   The other food proteins would then

TABLE II

*Relative Nutritive Value of Blood Proteins As Revealed by Their Amino Acid
Constitution*

	Whole egg proteins	Percentage deviation from whole egg values			
		Whole serum	Serum albumin	Fibrin	γ-Globulin
	per cent				
Arginine....................	6.4	−5	−6	+22	−25
Histidine...................	2.1	+48	+67	+29	+19
Lysine.....................	7.2	+33	+44	+22	−7
Tyrosine...................	4.5	+20	+18	+29	+51
Phenylalanine..............	6.3	−14	+22	−5	
Tryptophane...............	1.5	+13	−80	+127	+93
Cystine....................	2.4	+54	+171	−21	+29
Methionine................	4.1	−54	−68	−24	−73
Cystine + methionine.......	6.5	−14	+5	−23	−35
Threonine.................	4.9	+29	+4	+61	
Leucine....................	9.2	+96	+29	+52	−12
Isoleucine.................	8.0	−62	−75	−37	−59
Valine.....................	7.3	−18	−4	−18	+38
Indicated limiting amino acid.....................		Isoleu-cine	Trypto-phane	Isoleu-cine	Methionine
Greatest deficit.............		62	80	37	73

follow in the order of the percentage deficits in their respective limiting
amino acids.

Table III was compiled to reveal such a relationship. The limiting amino
acids are those indicated by biological assay, or, in the absence of such in-
formation, they are identified as those acids present in the protein in the
least amount relative to whole egg.   The percentage deficits in the third

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[4] Recent improvements in the method relate to equalized feeding by paired rats
on comparative diets, the use of a feces marker, and apportioning the endogenous
urinary N in proportion to the three-fourths power of the body weight rather than
with body weight itself.

column are taken directly from Table I.   The biological values and digestibilities of protein, occupying the next two columns, are taken from published and unpublished data secured in the Division of Animal Nutrition of the University of Illinois, except for lactalbumin, rice, sesame seed, and

TABLE III

Relation between Percentage Deficits in Limiting Essential Amino Acids with Reference to Whole Egg Proteins, and Biological Values of Proteins

Protein source	Limiting essential amino acid	Percentage deficit	Biological value	Digestibility	Bibliographic reference No.
			per cent	per cent	
Beef muscle.........	Cystine + methionine	29	76	100	(35)
" liver...........	Isoleucine	30	77	97	(36)
Egg albumin........	Lysine	31	82	100	(7)
Cow's milk.........	Cystine + methionine	32	90	95	(12)
Lactalbumin........	Methionine	34	84	98	(21)
Beef kidney........	Cystine + methionine	35	77	99	(36)
" heart	Isoleucine	35	74	100	(36)
Casein..............	Cystine + methionine	42	73	99	(20)
Sunflower seed......	Lysine	47	65	94	(37)
Soy bean...	Methionine	51	75*	96*	(37)
Rolled oats.........	Lysine	54	66	93	†
Yeast, average......	Cystine + methionine	55	69	93	†
White rice	Lysine	56	66	78	(38)
Corn germ	Methionine	61	78	85	(39)
Sesame seed.........	Lysine	61	71	92	(40)
Wheat germ.........	Isoleucine	62	75	95	†
Whole wheat........	Lysine	63	70	91	(41)
Cottonseed.........	"	63	61	90	†
Whole corn.........	"	72	60	94	(42)
White flour	"	72	52	100	(7)
Peanut.............	Methionine	76	58	97	(35)
Pea.................	"	76	48	91	(43)
Gelatin	Tryptophane	100	25‡	95‡	(44)

* The biological data were secured with heated soy flour.

† The digestibilities and biological values given are from unpublished data secured on growing rats in the Division of Animal Nutrition, University of Illinois.

‡ The biological data were secured with "pork cracklings" consisting essentially of connective tissue.

the pea.   The literature references in the last column of Table III denote the source of the biological data.

Inspection of Table III reveals a correlation between the chemical and the biological evaluations of food proteins in that the lower biological values

tend to gravitate toward the foot of the table, while the better proteins are found at the top.

The correlation coefficient of percentage deficits in limiting essential amino acids and biological values is -0.861 by the product-moment method, in which perfect negative correlation is represented by -1.000. It is worthy of note that little or no correlation exists between amino acid deficits and coefficients of digestibility, with $r = -0.366$. If the latter correlation is a significant one, it exists, not because of any inherent relationship between the content of proteins in essential amino acids and their
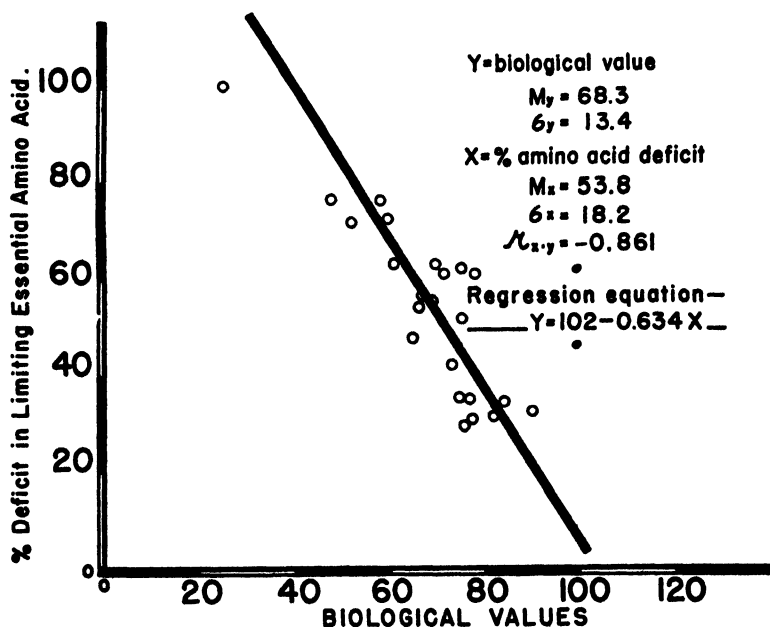
FIG. 1. Correlation between the chemical constitution and the biological values of proteins.

digestibility by enzymes, but because of the association of plant proteins of lower biological value with polysaccharides such as celluloses and hemicelluloses that as Mendel and Fine (45) showed many years ago, impair proteolysis by imposing indigestible barriers between protease and substrate.

The degree of correlation between the percentage deficits in essential amino acids of proteins and their biological values, as was determined by the nitrogen metabolism method, is shown graphically in Fig. 1. The regression equation for the prediction of biological value (y) from the maximum percentage deficit in essential amino acid (x) is $y = 102 - 0.634x$. This equation means that with zero deficit the biological value should be

100, the difference between 102 and 100 being an error of random sampling. For a percentage deficit of 100, the biological value should be 39. It seems reasonable to qualify this latter statement to apply only to proteins whose biological efficiency is limited by the complete absence of an amino acid essential for growth but not for maintenance, such as histidine. Other-- wise, the replacement of endogenous losses is impaired, as well as the ability to support growth, so that the biological value possesses a somewhat dif- ferent significance. With gelatin, tryptophane, the first limiting amino acid, is required for maintenance as well as for growth, and the biological value of 25 is for this reason, perhaps, somewhat less than the prediction, 39.

Hegsted, Hay, and Stare (29) compared the growth-promoting value of serum albumin, fibrin, and γ-globulin from human plasma with young rats fed *ad libitum* in rations containing 20 per cent of protein. The albumin proved to be the poorest, the fibrin definitely and markedly the best, almost as good as the proteins of skim milk powder, and γ-globulin was of inter- mediate value. The maximum percentage deficits in essential amino acids for these proteins, given on the bottom line of Table II, agree with this biological evaluation, the deficits being 37 for fibrin, not much more than the 32 for milk proteins given in Table III, 73 for γ-globulin, and 80 for albumin.

There are obvious imperfections in the correlation of chemical and bio- logical data summarized in Table III. These imperfections may in part be traceable ultimately to inaccuracies in the data. The average biological values determined in the Nutrition Laboratory of the University of Illinois will have a standard error of about 1.2 (35). The percentage deficits in limiting amino acids, being difference values, may be subject to a much greater error. But there are other disturbing factors in the picture. The biological values relate to the total nitrogen content of the food material, while the amino acid analyses may not. In Table III the animal tissues, muscle, liver, kidney, and heart, rank higher on the chemical scale than on the biological scale. All of these tissues contain considerable amounts of non-protein nitrogenous substances possessing little value in relation to the animal functions that dietary protein serves. The biological values of the true proteins in these tissues may be appreciably higher than those of the conventional proteins (N × 6.25).

Wheat germ and corn germ proteins, on the other hand, are rated much lower on the basis of their chemical structure than on that of their biological performance. The explanation is not at all obvious. The high nutritive value for peanut protein that has been secured by another method of bio- logical assay than the biological value in the sense of Thomas (46) finds no support from the chemical data reported in Table I.

Another possibility is that an imperfect correlation actually describes the

situation, other factors than the amino acid constitution of proteins being involved in their nutritive availability. One illustration of the operation of such factors is afforded by the changes occurring in seed proteins on germination. Everson *et al.* (47) showed that germination of soy beans greatly improved their nutritive value for growing rats, as measured by the gain secured per gm. of protein consumed, without improving the digestibility of nitrogen. This observation was confirmed for rats, but not for chicks, by Mattingly and Bird (48). However, Block and Bolling (6), by analyses for tyrosine, tryptophane, phenylalanine, cystine, and methionine, were unable to detect any change in soy bean protein during germination for 96 hours. Since the amino acids limiting the biological value of soy bean proteins are cystine and methionine, their constant proportion in the protein molecule during germination would not lead one to expect any change in nutritive value.

Effect of Heat on Proteins[5]

When food products are heated, their proteins are known to undergo certain changes in nutritive value. The digestibility may be improved (49) or depressed (12) and the biological value may be similarly changed. The improvement in the digestibility and the biological value of certain of the legume proteins is a striking phenomenon (50, 37). Soy bean proteins have been studied most thoroughly in this connection, and it has been shown that the raw, as well as the heated, proteins are deficient in the sulfur-containing amino acids. In some way, heat renders these amino acids more available in nutrition, obviously without changing the content of essential amino acids. The report of Ham, Sandstedt, and Mussehl (51) that the application of heat to soy beans destroys a proteolytic-inhibiting substance in the raw bean may partially explain this phenomenon. The position of soy bean proteins in the rankings attempted in Table III supports a correlation between limiting amino acid deficiencies and biological value only when the maximum biological value of the heated product is considered. The raw product, with a biological value of 59 (37), would be distinctly out of line with the other proteins. Evidently the application of heat is necessary to attain the full potential nutritive capacity of soy bean proteins.

The protein of the pea possesses a low biological value and its chemical rating is also low (see Table III). On heating, its nutritive value is depressed (19). The cereal proteins and the proteins of milk, which also are impaired by heating, each seem to have comparable chemical and biological ratings. On the basis of these facts, one might be tempted to venture the prediction that, in such a listing as is illustrated in Table III, food products

whose unheated proteins are ranked much lower by an adequate biological assay than by a chemical appraisal of the type here used would exhibit an improvement in biological value on heating, while those food proteins whose biological assays and chemical ratings show reasonable agreement would exhibit a decrease in biological value on heating.

The most usual effect of heat on the nutritive qualities of food proteins is a depressing one, well illustrated by the recent work of Stewart, Hensley, and Peters (52) on oats and mixed cereals.

We would like to present a somewhat intensive study of the changes that occur in cereal proteins during heating, first, with reference to their digestibility and biological value for the immature and the mature rat, and, then, with reference to the content of raw and heated proteins in the essential amino acids.

The cereals studied were an oat-corn-rye mixture sold as a breakfast food,[6] and rolled oats. The method of assay of protein (nitrogen) digestibility and biological value was essentially that described by Mitchell (34),[4] with young growing albino rats, and, later, mature rats.

The average digestibilities and biological values of the nitrogen of the unheated, the partially processed (pelleted), and the fully processed (exploded) cereal mixture are given in Table IV, together with the results obtained with raw and cooked rolled oats. The differences between average results, with their standard errors, are as follows:

	Differences with standard errors	
	Digestibility	Biological value
Cereal mixture, unprocessed *vs.* pelleted	2.84 ± 0.74	0.42 ± 0.74
Cereal mixture pelleted *vs.* fully processed	3.58 ± 0.72	11.47 ± 1.23
Cereal mixture, unprocessed *vs.* raw rolled oats	0.63 ± 0.71	2.10 ± 0.73
Rolled oats, raw *vs.* cooked	1.63 ± 0.68	2.86 ± 1.00

Such an analysis reveals that the pelleting of the cereal mixture definitely depressed the digestibility of the protein, without appreciably affecting its biological value. Further processing, involving treatment under high steam pressure (gun explosion), definitely and considerably lowered both the digestibility and the biological value of the protein. The raw rolled oats contained protein definitely, if only slightly, superior in biological value to the protein of the oat-corn-rye mixture, though no more digestible. Cooking the rolled oats in accordance with the recommended domestic practice probably lowers the digestibility of the protein, and increases slightly the biological value.

[6] Puffed oat cereal No. 1.

The data reported in Tables V, VI, and VII were obtained with growing rats in a succession of three experimental periods, the standardizing period

TABLE IV

True Digestibility and Biological Value of Nitrogen of Cereal Products before and after Processing, for Growing Rats*

Products	No. of determinations	True digestibility	Biological value
		per cent	*per cent*
Oat-corn-rye mixture, unprocessed........	12	91.67 ± 0.56	62.67 ± 0.48
" " pelleted............	12	88.83 ± 0.49	62.25 ± 0.56
" " † fully processed....	24	85.25 ± 0.53	50.78‡ ± 1.10
Rolled oats, raw........................	30	92.30 ± 0.43	64.77 ± 0.55
" " cooked......................	24	90.67 ± 0.53	67.63 ± 0.83

* These determinations were carried out in the research laboratories of General Mills, Inc., by Miss Claire A. Frederick under the supervision of Dr. C. G. Ferrari.

† Puffed oat cereal No. 1.

‡ Average of eighteen determinations.

TABLE V

True Digestibility and Biological Value for Growing Rats of Nitrogen of Oat-Malted Wheat Flour Mixture before and after Heat Processing (Gun Explosion)

Rat No.	Period No.	Processed mixture*		Period No.	Unprocessed mixture	
		True digestibility	Biological value		True digestibility	Biological value
		per cent	*per cent*		*per cent*	*per cent*
95 M.	1	86	53	3	90	64
97 "	1	83	50	3	91	64
99 "	1	87	53	3	94	65
101 F.	1	88	53	3	91	65
103 "	1	85	51	3	94	70
96 M.	·3	83	53	1	91	70
98 "	3	83	51	1	92	64
100 "	3	84	53	1	92	70
102 F.	3	80	49	1	94	64
104 "	3	82	52	1	90	59
Averages............		84.1	51.8		91.9	65.5

* Puffed oat cereal No. 2.

being the second, while the first and third periods were planned so that each rat received each of the test foods, half of them in one order and half in the reverse order (for further details of the procedure, see (37)).

TABLE VI

True Digestibility and Biological Value for Growing Rats of Nitrogen of Processed Oat-Malted Wheat Flour Mixture* and of Rolled Oats

Rat No.	Period No.	Oat-malted wheat flour mixture		Period No.	Rolled oats	
		True digestibility	Biological value		True digestibility	Biological value
		per cent	*per cent*		*per cent*	*per cent*
55 M.	1	83	51	3	90	69
57 "	1	84	48	3	91	65
59 "	1	88	50	3	93	70
61 F.	1	84	48	3	94	68
63 "	1	87	45	3	97	73
56 M.	3	90	52	1	91	65
58 "	3	87	50	1	94	67
60 "	3	89	54	1	91	67
62 F.	3	87	55	1	91	58
64 "	3	89	56	1	97	58
Averages...........		86.8	50.9		92.9	66.0

*Puffed oat cereal No. 2.

TABLE VII

True Digestibility and Biological Value for Growing Rats of Nitrogen of Uncooked Rolled Oats and of Oat-Corn-Rye Mixture*

Rat No.	Period No.	Rolled oats		Period No.	Oat-corn-rye mixture	
		True digestibility	Biological value		True digestibility	Biological value
		per cent	*per cent*		*per cent*	*per cent*
115 M.	1	91	67	3	90	63
117 "	1	89	71	3	92	63
119 F.	1	90	73	3	91	62
121 "	1	93	68	3	92	63
123 M.	1	90	67	3	93	62
125 "	1	91	65	3	93	64
116 "	3	90	67	1	91	64
118 "	3	91	66	1	93	64
120 F.	3	90	68	1	92	67
122 "	3	89	69	1	94	71
124 M.	3	91	67	1	92	63
126 "	3	92	67	1	93	63
Averages...........		90.6	67.9		92.2	64.1

* Puffed oat cereal No. 1.

With a slightly modified cereal mixture consisting mainly of oats, it is apparent from Table V that the gun explosion process lowered the digestibility of the proteins by 7.8 percentage units and the biological value by 13.7 percentage units. The proteins of the processed cereal mixture were also inferior to the proteins of rolled oats, both in digestibility and in biological value (Table VI). The oat-corn-rye mixture, unprocessed, contained

TABLE VIII

Replacement Value of Nitrogen of Processed Oat-Corn-Rye Mixture* on That of Unprocessed Mixture for Adult Male Rats, Comparing Each Rat with Its Pair Mate in Same Experimental Period

Period No.	Rat No.	Source of protein	Nitrogen intake per day	Nitrogen balance per day	Difference in nitrogen balance	Replacement value
			mg.	*mg.*	*mg.*	*per cent*
1	21	Processed mix	119	+2.31		
	22	Unprocessed mix	120	+15.58	13.27	89
	23	Processed mix	119	+3.86		
	24	Unprocessed mix	120	+22.58	18.72	84
	25	Processed mix	119	−4.66		
	26	Unprocessed mix	120	+25.64	30.30	75
	27	Processed mix	119	+1.63		
	28	Unprocessed mix	120	+15.06	13.43	89
	29	Processed mix	127	+2.44		
	30	Unprocessed mix	128	−2.91	−5.35	104
2	21	Unprocessed mix	84	+9.57	18.81	78
	22	Processed mix	84	−9.24		
	23	Unprocessed mix	84	+6.59	14.90	82
	24	Processed mix	84	−8.31		
	25	Unprocessed mix	84	−2.00	6.75	92
	26	·Processed mix	84	−8.75		
	27	Unprocessed mix	84	+1.82	9.98	88
	28	Processed mix	84	−8.16		
	29	Unprocessed mix	90	+1.66	13.64	85
	30	Processed mix	91	−11.98		
Average...................						86.6

* Puffed oat cereal No. 1.

somewhat more digestible proteins than rolled oats, but with a somewhat inferior biological value (Table VII).

Additional evidence of the injurious effects of the gun explosion process on the nutritive value of the proteins of cereals is afforded by the data collected in Table VIII on the nitrogen metabolism of mature albino rats. In these experiments, the rations contained only 4 or 5 per cent of the test proteins, as contrasted with the tests on the growing rats, which received

rations containing about 10 per cent of protein. The lower protein requirements of maturity, as compared with adolescence, dictated such a change. In the two experimental periods, the diets were reversed for all rats, and a system of paired feeding was adopted whereby paired rats received equal amounts of their respective diets. The nutritive efficiency of the comparative proteins was judged entirely on the basis of the nitrogen intake and the balance of nitrogen. The replacement values of processed on unprocessed cereal proteins were computed in accordance with the scheme proposed by Murlin, Nasset, and Marsh (53); namely, 100 minus the difference in nitrogen balance on the two test proteins expressed as a percentage of the intake of nitrogen. In the present case, a replacement value less than 100 indicates the inferiority of the processed protein.

In nine of the ten comparisons presented in Table VIII, the processed protein proved to be inferior to the unprocessed protein. The average replacement value was 86.6, indicating a heat damage of 13.4 per cent. Inspection of the complete data of this experiment reveals that this damage results in about equal degree from an impairment in digestibility and an impairment in metabolic utilization, as measured by the biological value.

The data presented above reveal a marked depression in the digestibility and the biological value of the proteins of cereal mixtures subjected to the extreme heat of the gun explosion process of breakfast food manufacture. This depression is evident in the nutrition of maturity, as well as in adolescent nutrition. However, when these "puffed" cereals are consumed with the usual proportions of milk, the nutritive value of the mixed proteins is high, owing to the marked supplementary relations existing between the proteins of cereals and of milk.[7]

The data also reveal that oat protein, subjected to domestic cooking, is not impaired in nutritive value, but that the protein of uncooked rolled oats is definitely superior to that of the cereal mixtures tested, whether processed or unprocessed.

In order to determine whether these changes in nutritive value of cereal proteins were associated with changes in their contents of the essential amino acids, analyses for the latter were carried out by one of us (R. J. B.) upon the unprocessed, the pelleted, and the exploded cereal mixture and upon uncooked rolled oats. The results are assembled in Table IX. The methods used in these analyses are indicated in Table X. These methods were the same as those used to obtain the amino acid content of whole egg

[7] Thus, the heat-damaged cereal mixture, for which data are reported in Table V, when consumed (by growing rats) with a 1:1 milk-cream mixture in the proportion of 1 part of dry cereal to 4 parts of milk-cream, exhibited a protein digestibility of 93 per cent and a biological value of 85. These values apply, of course, to the mixed proteins.

TABLE IX

Amino Acid Content of Processed and Unprocessed Cereals; All Values Calculated to Protein Containing 16 Per Cent Nitrogen

Amino acid	Oat-Corn-Rye Mixture*			Rolled oats
	Unprocessed	Pelleted	Pelleted and exploded	
	per cent	*per cent*	*per cent*	*per cent*
Arginine..............	5.0	5.4	5.0	5.8
Histidine..............	1.9	2.0	2.1	1.9
Lysine................	2.1	2.0	2.2	1.9
Tyrosine..............	4.3	4.1	4.0	4.1
Phenylalanine.........	5.6	5.5	6.0	5.4
Tryptophane..........	1.1	1.1	1.1	1.1
Cystine...............	1.6	1.7	1.5	1.4
Methionine............	2.4	2.4	2.5	2.1
Threonine.............	3.6	3.9	3.5	3.2
Leucine...............	8.8	8.7	8.8	8.9
Isoleucine.............	5.6	5.4	5.4	4.9
Valine................	6.2	6.0	5.8	5.5

' Steps in the manufacture of puffed oat cereal No. 1.

TABLE X

Amino Acid Methods Employed

Amino acid	Method	Type of hydrolysis	No. of hydro-lyses	No. of replicate determinations
Arginine..........	Kossel-Block isolation	8 N H_2SO_4	4	4
Histidine........	" "	8 " "	4	4
Lysine..........	" "	8 " "	4	4
Tyrosine........	Millon-Lugg colorimetric	5 " NaOH	2	8
Phenylalanine...	Kapeller-Adler "	5 " "	2	12
Tryptophane....	Millon-Lugg "	5 " "	2	8
Cystine.........	Fleming-Vassel "	Formic acid-HCl	2	8
Methionine......	McCarthy-Sullivan colori-metric	18% HCl	2	6
Threonine.......	Block-Nicolet oxidation	18 " "	2	6
Leucine.........	Microbiological	3 N "	2	20
Isoleucine.......	"	3 " "	2	20
Valine..........	"	3 " "	2	20

proteins (Table I), with the following exceptions: threonine was determined by the oxidation method of Shinn and Nicolet (54), and lysine by the microbiological method of Dunn *et al.* (55), as well as by isolation as the picrate.

It will be noted from the data in Table IX that the amino acid content of

the proteins of the oat-corn-rye mixture has not been altered appreciably, either by the heat involved in the pelleting process or by the more severe heat used in the gun explosion process. In particular, the lysine content shows no evidence of impairment, a fact of interest because lysine is the amino acid limiting the nutritive value of cereal proteins. Here we have, therefore, a definite impairment by heat of the digestibility and biological value of the proteins in a cereal mixture (Tables V and VIII), with no apparent destruction of the essential amino acid, lysine, limiting the biological utilization of the proteins (Table IX). Block, Jones, and Gersdorff (56) showed that the lysine content of casein was not impaired by exposure to dry heat at a temperature of 150°. It was suggested that this treatment may have brought about a molecular rearrangement "so that a part of the lysine precursors (rests) become resistant to enzymatic degradation." The formation of a new peptide linkage between the ε-amino group of lysine and a free carboxyl group of other amino acids is a possibility.

The data of Table IX fail to suggest, much less indicate, any essential difference in the protein value of rolled oats and of the oat-corn-rye mixture, although the results of the test on immature rats presented in Table VII reveal a very distinct superiority in nutrition of the proteins of oats over those of the mixture, and Table VI presents evidence on growing rats of the superiority of oat proteins over those of a similar cereal mixture. Possibly those differences in biological value are well within the analytical error of amino acid methods.

A depression of the nutritive value of proteins by heat without involving amino acid destruction is conceivable on the following grounds. (1) The digestibility of the protein may be depressed without incurring amino acid destruction, as Seegers and Mattill (57) found for heated liver preparations. (2) A decreased digestibility may involve the elimination in the feces of a protein fraction containing disproportionate amounts of certain amino acids, as Jones and Waterman (58) found for the protein, arachin. (3) The application of heat to a protein may promote certain combinations between terminal groupings that are resistant to proteolytic action, resulting in atypical peptides that may be absorbed as such (59, 60) and excreted in the urine.

SUMMARY

1. The relationship of the amino acid constitution of a protein, or of the protein component of a food product, to its nutritive value for the growing rat can be best revealed, in the absence of accurate values for the amino acid requirements, by computing for each protein, or protein moiety, the percentage deviations of the contents of each essential amino acid, expressed per 16 gm. of nitrogen, from the corresponding contents of a protein mix-

ture, such as that of whole egg, that is almost completely digestible by the rat and utilizable in adolescent metabolism. This has been done for a series of twenty-eight proteins and protein mixtures for which satisfactory analyses have been secured for all of the essential amino acids.

2. From such computations, the essential amino acid limiting the nutritive efficiency of the protein will be revealed as that one whose percentage deficit from that of the standard protein (whole egg) is the greatest, due consideration being given to the reciprocal relation existing between cystine and methionine in anabolism. The limiting amino acids thus indicated agree with those determined in feeding experiments with only one or two exceptions.

3. The proteins of foods may be ranked in the order of decreasing nutritive efficiency on the basis of increasing percentage deficits (as above defined) in their respective limiting essential amino acids. These percentage deficits are highly correlated ($r = -0.86$) with the corresponding biological values determined by the nitrogen metabolism method. Little or no correlation exists between the chemical ratings of the proteins and their digestibility by the growing rat.

4. The biological value of a protein (y) may be roughly estimated from its maximum percentage deficit in an essential amino acid (x) by the equation: $y = 102 - 0.634x$.

5. However, there are known instances in which the biological value of a protein, or protein mixture, and its chemical rating do not agree, for various reasons discussed in the text.

6. In particular, the nutritive value of cereal proteins may be greatly impaired by the application of heat with no demonstrated alteration in their content of the essential amino acids.

7. A basis for predicting the effect of heat on the biological value of a food protein is suggested.

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CHANGES IN THE INORGANIC CONSTITUENTS OF DEVELOPING SALMON EGGS*

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Changes in the distribution of sodium, potassium, calcium, magnesium, chloride, and phosphorus have been followed during the early development of *Salmo salar*. Needham (1) gives the constitution of undeveloped fish eggs; the literature on changes during development is referred to below.

A sample of unfertilized celomic eggs from one fish was bottled in celomic fluid for analysis, and the rest of the eggs from this female were moved from the hatchery to the laboratory 2 days after fertilization and reared there. The analyses given below of celomic fluid, celomic eggs, and water-hardened eggs 2 days after fertilization all refer to products of this same parent salmon. When the eggs from the first fish were used up, those from a second one were brought in, and still later eggs from a third female were used, the work being continued up to the time of complete absorption of the yolk sac. The first lot averaged 118 mg. in celomic fluid and 138 mg. when developing; results from the second and third lots have been recalculated to this basis. Weights of yolks and embryos were obtained in part by direct weighing and in part from unpublished data of Armstrong. Yolk is taken as 59 per cent water and embryo as 86 per cent water (2). Fertilization has been called −50 days and the central hatching date zero; intermediate times were recalculated accordingly. In placing the batches with reference to one another, use was made of the stages described by Pelluet (3).

Methods

Analyses were made of the whole eggs, larvae, yolks, and embryos. A larva is an embryo with the yolk sac attached or, expressed differently, the whole egg minus capsule and perivitelline fluid. Dissection of the embryo from the yolk sac was first practicable at −18 days.

Preparation of Samples—Wet ashing with nitric acid in a Kjeldahl flask (4) was successful but required 50 hours. A great shortening of time was made possible by the perchloric-nitric method of King (5). As an alternative to ashing, trichloroacetic acid extracts were made. The material was first frozen on sand in a mortar; it was then ground

until smooth, with gradual addition of acid, and quantitatively transferred to a centrifuge bottle, shaken for 10 minutes, centrifuged, and filtered, the resultant clear solution being used for analysis of all four metals as well as phosphorus. Usually 50 to 100 specimens were subjected to ashing or extraction.

Potassium—The method followed was that of Kramer (6), ceric sulfate being used in the final titration (7).

Sodium—The procedure was basically that of Ball and Sadusk (8), suggested modifications (9) being found unnecessary.

Magnesium—The method was that of Greenberg *et al.* (10, 11), calcium and iron being removed as a preliminary.

Calcium—The method of Larson and Greenberg (12) was followed, certain modifications (13, 14) having been tried and found unnecessary.

Chloride—The method of Van Slyke (15) was used.

Phosphorus—For the inorganic fraction the trichloroacetic acid extract was used; total phosphorus was determined in dissolved wet ash. The colorimetric method of Fiske and Subbarow (16) was followed.

Bicarbonate—The bicarbonate value of the celomic fluid was kindly obtained by Dr. E. C. Black, for whose methods see earlier papers (17, 18). All of the CO_2 was calculated as bicarbonate. As to eggs, the bicarbonate figure for yolk was obtained from data for *Salvelinus fontinalis* of Irving and Manery (19) who found 1.39 volumes of CO_2 per cent wet weight during earliest development, half of which was bicarbonate. Taking an egg as 67 per cent water (20) and dividing by the gm. molecular volume, one obtains

$$\frac{1.39}{2} \times \frac{100}{67} \times \frac{10}{22.4} = 0.5$$

milliequivalent of bicarbonate per kilo of egg water, a negligible amount.

Celomic Eggs and Celomic Fluid

The ovarian or celomic fluid, which appears rather suddenly just at the time of hatching (21), is clear, limpid, and slightly translucent. It has 1.30 per cent of solids, a specific gravity of 1.0070 at $20°/4°$, and contains, in milliequivalents per kilo of water, Na 151, K 3.2, Ca 7.1, Mg 2.6, Cl 116, HPO_4 4.0, HCO_3 13.4. In the Salmonidae the ripe eggs lie freely in the celom. Fig. 1 compares celomic fluid with the eggs in it, the latter being called "early yolk."

The conclusions to be drawn from Fig. 1 are that *in the celomic fluid* (1) sodium is the dominant base, and chloride is the dominant acid; (2) the base found is 31 milliequivalents in excess of the acid found, which indicates that protein is used for binding base; (3) the relative distribution of ions

is in accord with analyses of sea water and vertebrate serum (22); *in the celomic eggs* (4) potassium is the dominant base, but large proportions of calcium, magnesium, and sodium are found as well; (5) there is a large excess of base over acid, namely 204 milliequivalents, to be accounted for by assuming that some of the protein is used for base-binding, and that most of the Ca and Mg is not ionically active (see below).

The freezing point depressions, Δ, of celomic eggs and celomic fluid have been calculated from base to see whether the analyses are in general agreement with Δ as directly observed. Some values from the literature for
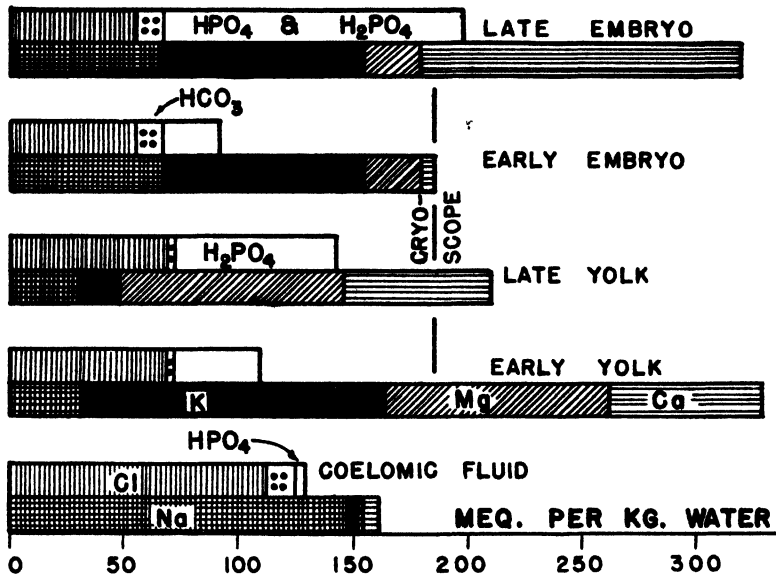
FIG. 1. A comparison of the ionic constitution of the celomic fluid, yolk, and embryo. The cryoscopic line is based on data from the literature. Only inorganic phosphorus is considered, and it has been assigned a valence of 2 in the celomic fluid, 1 in the yolk, and 1.8 in the embryo.

this quantity are assembled in Table I together with our own calculations. From Table I the following conclusions may be drawn: (1) Ripe eggs in the celom are somewhat hypertonic to the blood (25, 26), while the celomic fluid is markedly hypotonic to blood (21, 23, 24). The follicular fluid of the cow and pig is also hypotonic to blood (27). (2) The total base of the celomic fluid accounts correctly, on assumptions made, for the osmotic pressure reported in the literature, while the total base of the egg, when assumed to be osmotically active, gives results that are one-third higher than reported cryoscopic measurements (present work). (3) If in the eggs only Na and K are considered as participating in osmotic activity,

the calculated Δ is only slightly lower than the observed Δ (present work). If it is assumed, as is usual, that all or nearly all of the Na and K is active, it follows that only a small portion, 10 to 15 per cent, of the Ca and Mg is osmotically active, the remainder being organically bound.

Schmidt-Nielsen and Schmidt-Nielsen (26) found that the juice from cooked salmon muscle gave a Δ value of 0.994, or about the same as our calculated total base value for eggs. Some bound salt was doubtless liberated by the boiling. In the yolk of the hen's egg, Δ calculated from analyses is 1.22, while the observed value is only 0.60 (28). The value of Δ for chick yolk has been reported as even lower; namely, 0.42 to 0.45

<div align="center">

TABLE I

Comparison of Observed Values of Freezing Point Depression, Δ, of Anadromous Salmonids in Fresh Water at Spawning Time with Calculated Results of Present Analyses

</div>

		Blood	Celomic fluid	Eggs in celom
Salmo salar	Atkins (23), 1 female		0.560	
	This paper, calculated from total base		0.555	0.948
	This paper, calculated from Na and K only			0.599
" *salvelinus* (syn. *alpinus*)	Bogucki (24), both tests from same fish		0.615	0.640
	Runnström (25), whole blood and eggs from different fish	0.636		0.645
	Schmidt-Nielsen and Schmidt-Nielsen (26), average of 2; serum and eggs from same fishes	0.620		0.642
Oncorhynchus tschawytscha	Greene (21), average of 6 females; both tests on each; serum and whole blood the same	0.602	0.552	

(29, 30). For trout yolk it remains unchanged throughout development (31).

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Ionic Changes When Egg Is Laid

</div>

When an egg is laid, it is normally fertilized and at the same time water-hardening begins, the latter process being independent of fertilization. To the discussion given by Hayes and Armstrong (2) we now add in Table II the evidence of direct analysis. There is evidently a small loss of certain ions; the diminution in base would lower the osmotic pressure by about 3 per cent. As to anions, while we found an 8 per cent loss of chloride,

Manery and Irving (20) report that no loss occurs in *Salmo gairdneri* eggs. The other anion, inorganic phosphate, represents only one-quarter of the total phosphorus; so that a change might mean either loss from the egg or conversion into organic phosphorus.

The bottom line of Table II gives the only previous salmon egg analysis found (4). The results, except for calcium, åre somewhat lower than ours but show substantial agreement especially in ratio to one another.

Osmotic Relations of Yolk and Embryo

Fig. 1 is a barograph of conditions shortly after the beginning, and at the time the yolk is nearly used up. Except as otherwise noted the values are taken from the smoothed curves of Fig. 3. The line in Fig. 1 marked "cryoscope" is based on the data for *Salmo salvelinus* in Table I, which presumably apply to yolk after water-hardening, as well as before. It

TABLE II

Changes in Salt Concentration As Result of Transfer of Eggs from Celomic Fluid to Water

The values are in mg. per 118 mg. of yolk, which has 59 per cent water. The bottom line gives analyses of Bialaszewicz (4) on *Salmo salar* celomic eggs, adjusted to a 118 mg. yolk.

Material	Na	K	Ca	Mg	Cl	Inorganic P	Total P
Celomic egg..........................	0.067	0.363	0.084	0.081	0.195	0.096	
Fertilized egg in water...............	0.060	0.363	0.077	0.081	0.180	0.081	0.558
Per cent loss.........................	10	0	8	0	8	16	
Bialaszewicz.........................		0.283	0.122	0.053	0.141		0.411

shows the concentration of mixed base which corresponds to a freezing point depression of 0.64°. The extension of the cryoscope line across the embryo suggests that embryo and yolk are isotonic, which is probably true.

The large stores of calcium and magnesium in the yolk remain unchanged in concentration during development, but the potassium concentration diminishes so greatly that it is necessary to assume that most of the formerly inactive calcium and magnesium come to participate osmotically. The utilization of organic material for metabolic purposes would free salt and increase the inorganic phosphorus.

Bialaszewicz (32) developed an ingenious technique to separate the "dispersed phase" or colloidal phase with its non-participating inorganic constituents from the "intermicellar liquid." In *Salmo fontinalis* yolk he found that the following percentages of the substances named were organically bound, hence inactive: K 11, Na 49, Ca 73, Mg 68, Cl 0, total

P 90. His method is theoretically sound and the general conclusions warranted. The results, however, present quantitative difficulties which have been discussed by Krogh (33).

The early embryo has a quantity of base which agrees with cryoscopic tests and could be reasonably thought to be almost wholly active. The same would be true of the late embryo were it not for the striking increase in calcium and phosphorus, the excess of which must be inactive.
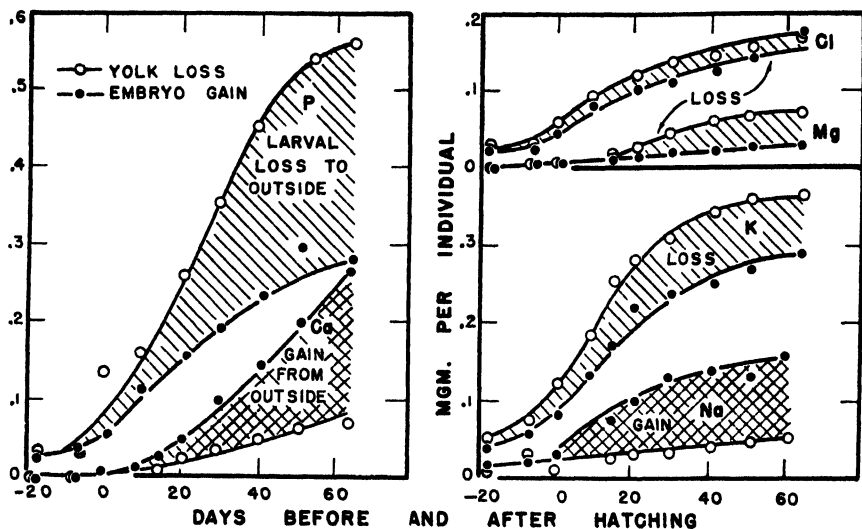
FIG. 2. A comparison of the loss of each mineral from the yolk with the gain by the embryo. When the embryo curve is above the yolk curve, an uptake from the surrounding water is indicated. This is so in the case of sodium and calcium. The other substances sustain loss during development. Fertilization occurred at −50 days.

Changes During Development

To illustrate the ionic trends Figs. 2 and 3 have been drawn. In Fig. 2 the yolk loss is obtained by subtracting the later weights from the initial yolk value; thus it was found that at −7 days the yolk contained 0.288 mg. of potassium. The initial store being 0.363 mg. (Table II), the point on Fig. 2 for −7 days is placed at (0.363−0.288) or 0.075 mg. The potassium of the embryo on that day was 0.055, which is plotted as "gain by embryo." In Fig. 3, dealing with concentrations, experimental points have been set down for those ions which appear to exhibit a trend, but where no trend was apparent only the average level has been placed on the chart without supporting points. The degree of scattering of points about

the horizontal lines is indicated by the following list, which shows the per cent probable errors.

	Yolk	Embryo
Sodium...	10	3
Potassium......................................		3
Calcium..	3	
Magnesium.....................................	3	10
Chloride.......................................	9	6
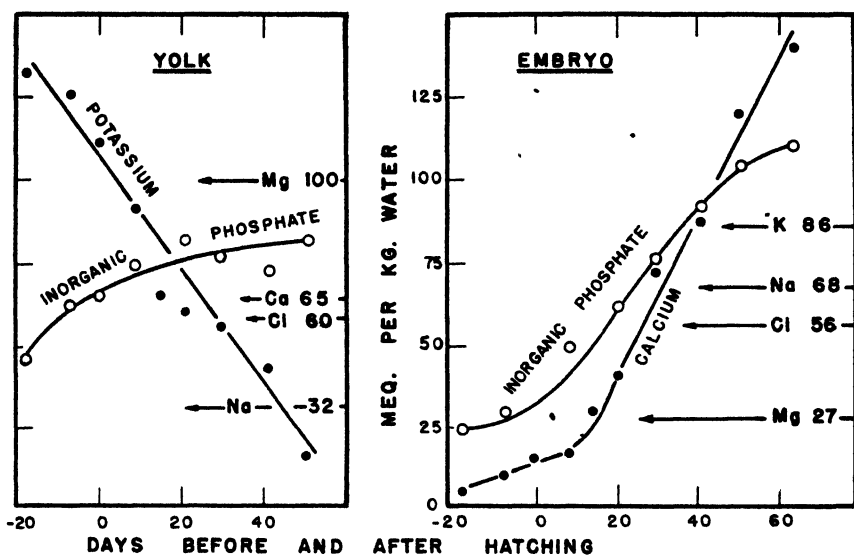
FIG. 3. Changes in mineral concentration in the yolk during development. The four elements to the right have been considered as constant, because no trend could be observed. For their fluctuations, which were considerable, see the text. Phosphorus has been assigned a valence of 1 in the yolk and 1.8 in the embryo.

It may be noted that the milliequivalents per kilo of water will be less accurate than mg. per specimen, since in the former there are combined with the analytical errors those of weight and water content.

Sodium—As Fig. 2 shows, there is an uptake of sodium from the surrounding water, which appears to begin rather suddenly at the time of hatching. By the time the yolk sac has disappeared the embryo contains over twice as much sodium as the egg had at the beginning (for which see Table II). In the matter of concentration, the fluctuations appear to be random, and it is concluded that the yolk maintains 32 and the embryo 68 milliequivalents per kilo of water.

Potassium—The concentration of potassium in the embryo shows no

trend but remains fixed at some 86 milliequivalents per kilo of water (Fig. 3). Leulier and Paulant (34) have followed potassium in rainbow trout larvae from the time of three-fourths absorption of the yolk sac until the young fish had a wet weight of 10 gm. They found a constant value only slightly higher than ours, some 95 to 100 milliequivalents per kilo of water. It thus appears that the level of potassium established at the earliest stages is permanently maintained.

The source of the embryonic potassium is the yolk, which starts out with 130 milliequivalents but is steadily denuded as development proceeds, until at the end potassium is the most scarce of the ions studied (Fig. 3). So far as our evidence goes, potassium is also the only ion for which a specific selective power by the embryo from the yolk can be demonstrated. Evidently the egg is provided with barely enough potassium to carry the embryo through to feeding time, but in spite of this, there is some loss to the outside, as Fig. 2 shows, so that the larval system is not wholly efficient in conserving its stores. Fig. 2 indicates that the loss begins before hatching, when the capsule becomes permeable (35). It is interesting that the developing larva, in spite of the shortage of potassium, is unable to absorb any from the water.

Calcium—Absolute calcium values are shown in Fig. 2. It will be seen that, beginning at about the time of hatching, the embryo gains a great deal more than the yolk loses, which represents uptake from the surrounding water. This finding confirms work on the brook trout by McCay *et al.* (36, 37) who found only 0.008 mg. of calcium per specimen in "green eggs," but 0.035 in fry with the yolk sac absorbed. When allowance is made for the difference in egg size, their results are a little over half as high as ours. In another paper (38), McCay and Tunison observed an uptake of calcium from the water in older trout and say, "This experiment shows clearly why we have failed to produce any disease resembling rickets in trout when we fed diets low in calcium and high in phosphorus. Our experiments do not indicate whether this calcium is absorbed through the gills or from the gastro-intestinal tract." As the foregoing statement suggests, the concentration of calcium in the embryo increases steadily as development proceeds (Fig. 3). The increase is paralleled by that of inorganic phosphate, so that these two materials form the dominant inorganic constituents of the embryo in later development. In the yolk the concentration of calcium shows only random fluctuations with a constant mean value of 65 milliequivalents per kilo of water (Fig. 3).

Magnesium—Concentrations (Fig. 3) remain constant at 100 and 27 milliequivalents per kilo of water for the yolk and embryo respectively. In absolute values (Fig. 2) there is a steady loss from the larval system, the egg being provided with twice as much as the embryo requires.

Chloride—The curves of Fig. 2 suggest that all of the loss occurs before hatching, and that there is no later loss or gain. Hence the embryo at the end contains almost as much chloride as the egg had at the start. As regards concentration, the embryo and yolk maintain constant and very nearly equal values throughout, namely 56 and 60 milliequivalents per kilo respectively. In a study of the trout egg as a whole, Manery and Irving (20) found a decline in chloride from some 60 milliequivalents per kilo of water in early stages, to 43 milliequivalents at the time of hatching, which is materially larger than that in the salmon.

Phosphorus—Results shown are probably approximately correct for yolk, but, for later embryonic values, a certain quantity of "labile organic phosphorus" in muscle may have been counted as inorganic P. On a test of a larva some time after hatching, 21 per cent of what is here called inorganic P turned out to be acid- and water-soluble organic P (method of Fiske and Subbarow (39)). Since the yolk is acid (40), we have here assumed a valence of 1. In the embryo, where the cells are approximately neutral and the intercellular fluids alkaline (41), the valence assigned is 1.8, as is given for mammalian body fluids which, as Shohl (42) points out, is merely a convention. *The larval system* shows a loss of phosphorus during development, probably beginning before hatching (Fig. 2). As against our finding here, McCay and Tunison (36) report that *Salvelinus fontinalis* takes up phosphorus during development, and even in early egg stages. They state, however, that "it is possible that the increases in the eggs are due to analytical errors" and, their larval stages having been fed, the values are not comparable to ours. They find at the start, allowing for difference in egg size, only one-half as much phosphorus as we do. *In the embryo*, inorganic P increases in concentration (Fig. 3), paralleling an increase in calcium. The marked rise begins some time after hatching, but it is not clear where the extra Ca and P go, for bone has not yet formed and cartilage does not contain excessive quantities (43); possibly calcified cartilage may be formed. Organic P was followed throughout development, showing an average value of 9.6 ± 0.7 gm. per kilo of wet weight. No concentration trend could be demonstrated. In the earliest observations there was, in mg. per embryo, 4 times as much organic as inorganic P, but, by the time the yolk had disappeared, the inorganic P had risen to nearly twice the organic. *In the yolk sac*, inorganic P appears to increase in concentration during development (Fig. 3). If the increase is genuine, it presumably means that a mechanism exists in the cells surrounding the yolk for breaking down the protein preparatory to transfer to the embryo and thus for liberating phosphate. The concentration of yolk organic P was found to remain at 8.8 ± 0.4 gm. per kilo of wet weight. This means that organic P disappears in proportion to the disappearance of

the yolk sac as a whole, a natural conclusion when it is remembered that the yolk sac (except for water) is largely protein and the organic phosphate is largely in the protein.   In mg. per specimen, the yolk inorganic P is only one-fifth of the organic at the start, but, by the end, it has risen to one-half.

Bicarbonate—The source of bicarbonate values for yolk in Fig. 1 has already been discussed.   For the embryo, the celomic fluid figure has been used.   It is assumed that the concentrations in the two phases are unchanged throughout development.   It appears that yolk bicarbonate is negligible in the salmonid, as in the chick (28).   Embryo bicarbonate is evidently a minor ion.

SUMMARY

Sodium, potassium, calcium, magnesium, chloride, and phosphorus have been estimated in celomic fluid, and followed in developing yolks and embryos of the salmon.   In the fluid bathing the ripe eggs, sodium chloride is dominant, with other ions approximately in the ratios found in sea water or serum.   In the eggs potassium dominates, but there are also large proportions of calcium, magnesium, and sodium.   Base found greatly exceeds acid found, leading to the view that most of the calcium and magnesium are not osmotically active.   Calculations of osmotic pressure suggest that celomic fluid is hypotonic to eggs.   Upon transfer from celomic fluid to water, at the time of fertilization, the egg loses minor quantities of sodium, chloride, and calcium, lowering the osmotic pressure of the yolk by about 3 per cent.   During development, the egg or larva takes up calcium and sodium from the environment, the final amounts being respectively 4 and 3 times the initial store.   Losses were observed in the other four elements, the most marked being phosphorus.   Loss of potassium began some time before hatching, thereby providing evidence for the permeability of the egg capsule.   Expressed as milliequivalents per kilo of water, the potassium of the yolk virtually disappears; inorganic phosphorus shows a small gain; other ions remain constant.   Experiments continued until the yolk was quite gone.   In the embryo, calcium and phosphorus concentrations increase to a marked degree, perhaps going to form the skeleton; other minerals exhibit no measurable trend.

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THE ADAPTATION OF THE BECKMAN SPECTROPHOTOMETER TO MEASUREMENTS ON MINUTE QUANTITIES OF BIOLOGICAL MATERIALS

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The smallest volume that has been used with commercial photocolorimeters is about 1 ml. The sensitivity and optical system of the Beckman spectrophotometer (model D or DU) are such that it can be readily adapted to volumes of the order of 50 c.mm. (0.05 ml.) without sacrificing length of the light path through the colored solution. With volumes of this order of magnitude the size of sample required is so small that it has been easily possible to devise methods for determining a number of substances of nutritional significance on a single sample of finger blood (*e.g.* vitamin A, carotene, ascorbic acid). Since it seems likely that colorimetry or spectrophotometry on this scale may prove useful on other occasions, particularly with biological material, when the size of sample is limited, the means of adaptation is described, together with some data illustrative of the precision which can be expected.

Principle

Samples are placed in a special cuvette (Fig. 1) which has the usual 1 cm. light path but which has a narrow inner width, of 2 mm. or less. 50 c.mm. of liquid will fill this cuvette to a height of about 2.5 mm., thus giving a column of the sample with a cross-sectional area of 2 × 2.5 mm. and a length of 1 cm. In front of the cuvette is placed a diaphragm which confines the light beam to a cross-section of less than 2 × 2 mm. This slender light beam can pass through the liquid without touching the cuvette walls or the liquid meniscus. In order to obtain sufficient light intensity for measurements when such a small area of the photocell is used, it is usually necessary to widen the spectral band employed; however, the band widths used are still narrow in comparison with those of most other photoelectric spectrophotometers or photocolorimeters. Alternatively, the use of a more sensitive galvanometer permits the use of narrower spectral widths than would otherwise be necessary.

Equipment

Cuvettes—These are square cells having inner dimensions of 2 × 10 mm. instead of the usual 10 × 10 mm. and are only 25 mm. high (Fig. 1).

Except for the height, the outer dimensions are the same as those of the standard cuvette. The light path is 10 mm., the same as for the regular cuvettes; however, the cross-sectional area is greatly reduced. (These cuvettes may be obtained in quartz from the Pyrocell Manufacturing Company, 207 East 84 Street, New York 28, New York.)

Smaller cuvettes (1 × 10 mm. inner dimension) requiring 30 c.mm. of liquid have also been used successfully, but, since their use is a little more difficult, it is recommended that the larger (2 mm.) cuvettes be given a trial before attempting to use the smaller ones.

Diaphragm—Two types (A and B) have been used and both have been found to be satisfactory. Type A can be home-made (Fig. 1). A piece of
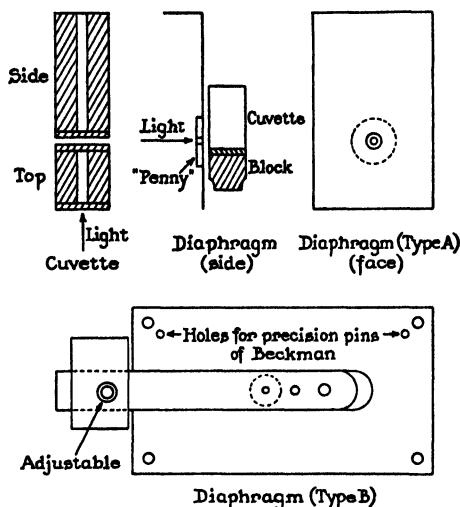
FIG. 1. Micro cuvette and diaphragms

metal of the size and shape of a penny is used. This must just fit the opening in the instrument, which leads the light beam toward the cuvette holder. A 1.0 to 1.4 mm. hole is drilled through the metal disk about 1 mm. off center. The disk is then held in the opening with a piece of Scotch tape and turned until the emergent pencil of light passes exactly in the middle between the two walls of the cuvette when in place in the carriage. The disk is now fastened at just this angle to a very flat thin piece of sheet steel. This sheet is about 6 cm. wide by 9 cm. long, with a 3 or 4 mm. hole 2.5 cm. from one end. The disk is soldered to the sheet so that the holes coincide and the long axis of the sheet is perpendicular. After soldering, the top of the sheet is bent at a right angle to form a flange which lies on the top of the instrument and keeps the sheet from turning. The cuvettes are now raised

on wooden blocks (Fig. 1) until the light beam just misses the bottom of the cuvette. (Special adjustable blocks may be obtained from the Pyrocell Manfacturing Company.) As much as possible of the "play" in the cuvette carriage assembly is taken out. The diaphragm may be inserted and removed by merely loosening the bolts holding the phototube housing. The cuvettes are ordinarily held close to one side of the carriage by a spring on the opposite side. When used for the micro cells and when the diaphragm is being adjusted, the carriage should be oriented so as to bring the cuvettes as near the diaphragm as possible, since the light beam spreads out a little after leaving the diaphragm, and the closer the cuvettes are to the diaphragm the less difference this will cause. The cuvettes are numbered and always inserted in the holder with the same orientation.

Type B, a somewhat more convenient and easily adjustable diaphragm attachment, may be obtained from the Pyrocell Manufacturing Company. The pinhole or pinholes are carried by a thin strip of brass (Fig. 1) which slides in a channel cut in a sheet of metal which is inserted in the spectrophotometer between the cuvette carriage assembly and the body of the instrument. The sheet of metal is held in exact position by holes drilled through it to match the precision pins of the instrument. After the metal sheet is inserted, the brass strip is brought to position by sliding it into the channel in the metal sheet until the pinhole coincides with the center of the cuvette. A stop on the brass strip is then adjusted with a special bolt so that the strip may always be brought to exactly this same position. . Provision is made so that either of two or three different sized pinholes may be brought into position without disturbing this adjustment. With this diaphragm the cells are raised on blocks, as described above. With the strip removed the instrument may be used with standard cuvettes without disturbing the metal sheet.

Procedure

Since there is usually a little "play" in the cuvette carriage, the cells should always be brought toward their position in front of the light from the same direction. If there are any traces of cement remaining on the cuvette walls, these should be removed with a needle, etc. The micro cuvettes are used like the larger cells except that, for convenience in handling, they are left mounted in the carriage. Samples are introduced with fine tipped pipettes into the bottom of the cuvettes, and the previous samples are removed by suction with a fine tipped glass tube attached to a suction pump or aspirator bottle. If the glass tip is slender, 99 per cent of the previous sample may be removed, thus eliminating the need for rinsing the cuvettes between analyses of a series unless the concentrations are widely different.

The 2 mm. cells require about 50 c.mm. of solution. The exact amount necessary should be determined by pipetting in solution until further addition is without effect. A large cell may be used in the first position in the holder, which is merely for adjusting the instrument to zero for the particular solvent or other blank being used. The small cells should give blank readings that differ by no more than about 0.01 on the density scale from the first (large) cell, and these blank readings should be reproducible to within 0.002 on the density scale.

TABLE I

Optical Density of Replicate Samples of Different Volumes Taken from Same Large Volumes

	0.0465 mg. per cent P			0.0186 mg. per cent P			0.00465 mg. per cent P		
	0.025 ml.	0.05 ml.	3 ml.	0.025 ml.	0.05 ml.	3 ml.	0.025 ml.	0.05 ml.	3 ml.
	Mg. P × 10^{-6}								
	11.6	23.2	1390	4.65	9.3	560	1.16	2.32	139
Optical density (870 mμ)	0.420	0.423	0.424	0.168	0.167	0.166	0.043	0.040	0.042
	0.420	0.422	0.424	0.166	0.165	0.166	0.043	0.043	0.043
	0.420	0.422	0.424	0.168	0.166	0.166	0.042	0.042	0.042
	0.421	0.422	0.423	0.167	0.165	0.165	0.043	0.045	0.042
	0.420	0.422	0.420	0.167	0.167	0.165	0.043	0.043	0.042
	0.425	0.420	0.424						
	0.422	0.423	0.424						
	0.421	0.421	0.424						
	0.419	0.421							
	0.422	0.422							
Average	0.421	0.422	0.423	0.167	0.166	0.166	0.043	0.043	0.042
S.D.	0.0017	0.0009	0.0014	0.0009	0.0010	0.0007	0.0005	0.0019	0.0005
Variance, %	0.4	0.2	0.3	0.5	0.6	0.4	1	4	1

With the early Beckman instruments a more sensitive galvanometer has been found to be advantageous for use with the micro attachment. The meter furnished with the instrument is replaced by one with a range of 200 microamperes. This is 5 or 10 times more sensitive than the original meter and has proved useful for macro as well as micro work. The sensitive meter is particularly valuable for use with the 1 mm. cuvettes or for measurements with larger cuvettes at wave-lengths at which otherwise insufficient light is obtained. Under these circumstances, it is sometimes necessary to adjust the instrument to zero at the "0.1" knob position. This allows the instrument to be used with one-tenth the ordinary amount of light, and, with the more sensitive meter, readings may be made as ac-

curately at this setting as when the instrument is adjusted to zero with the "check" position of the knob. With later instruments the original sensitivity is such that a more sensitive meter is not required.

DISCUSSION

Table I illustrates readings obtained at 870 mμ with 3, 0.05, and 0.025 ml. of solutions taken from three large volumes of inorganic phosphate
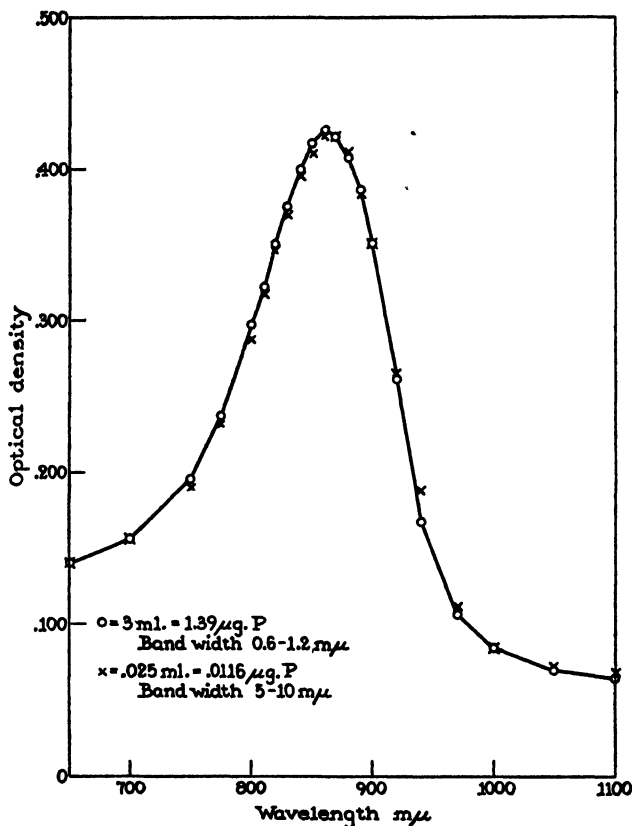
Fig. 2. Absorption curves of phosphomolybdous acid with 3 and 0.025 ml. volumes

of different strengths treated to form the blue phosphomolybdous color. (The color was developed with a modified Fiske and Subbarow reagent, 2.5 per cent ammonium molybdate, 0.6 per cent sodium bisulfite, 0.03 per cent sodium sulfite, and 4.5 mg. per cent of 1,4-aminonaphtholsulfonic acid in 1.2 N sulfuric acid, by heating 20 minutes at 100°.)

Fig. 2 gives the absorption curves obtained with the strongest of the above phosphate solutions (0.0465 mg. per cent). One curve was obtained

with 3 ml. of solution with the narrowest slits possible. The other curve was made with 0.025 ml. of solution (10^{-5} mg. of P) and necessarily a wider slit. The curves are so nearly superimposable that the second set of points was not connected in order to avoid confusion.

Fig. 3 gives a similar set of curves made with phenylalanine in the ultraviolet region. This compound was chosen since its spectrum contains a series of very narrow absorption bands. In this case the curves obtained
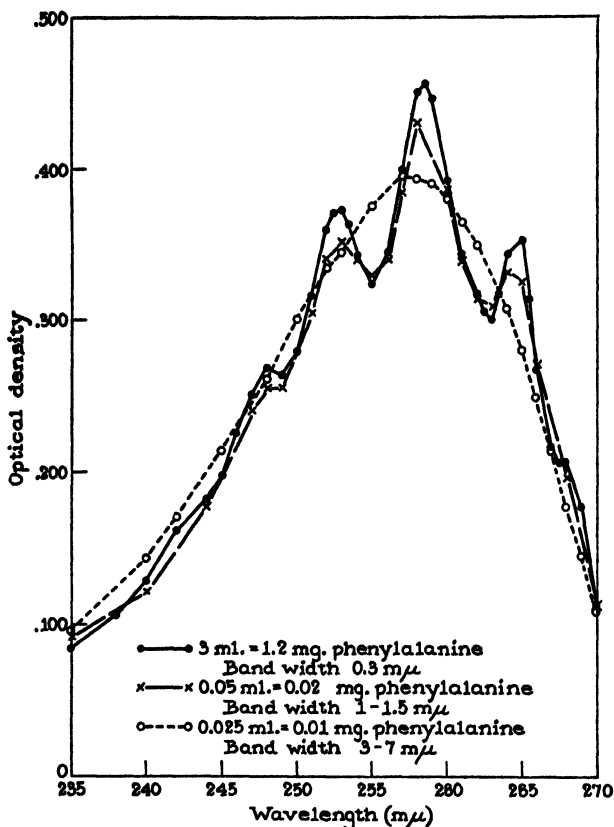
FIG. 3. Absorption curves of phenylalanine with 3, 0.05, and 0.025 ml. volumes

with 3, 0.05, and 0.025 ml. of solution are not superimposable, since with the wider slits required for the small volumes some of the fine detail is obscured. However, the difference between the 3 ml. and the 0.05 ml. curves is not marked. This illustrates a limitation which should be borne in mind if spectra with fine structure are to be measured. This difficulty, of course, would not interfere with *quantitative* measurements made even on compounds such as phenylalanine.

It is evident that satisfactory results may be obtained with the small volumes for both quantitative measurements and absorption curves. The 2 mm. cuvettes have proved to be as convenient to use in practice as the larger cells. Measurements may be made even more rapidly with the 50 c.mm. volumes than with larger samples.

Spectral curves can be made from about 235 to 935 mμ with spectral widths not exceeding 3 mμ with 25 c.mm. volumes, and from about 225 to 1050 mμ with spectral widths of not more than 3 mμ with 50 c.mm. volumes. With the type of adaptation described, it does not appear possible to utilize much smaller volumes than 25 c.mm., since with still narrower cells the setting of the carriage position would become too critical. It will probably be necessary to employ instead some other principle for adaptation to lesser volumes.

It has been felt desirable to use a fixed diaphragm with these cells instead of providing each cell with its own diaphragm which would then move with the cell. The disadvantage of such a movable diaphragm would be that a more refined carriage movement would be required. The light beam differs in brightness from point to point, as does the sensitivity of the phototube surface; hence minute differences in the point 'at which the carriage comes to rest would affect the phototube response.

With the adaptation described it has been possible to devise methods for the measurement of ascorbic acid in 0.01 ml. of serum,[1] vitamin A and carotene in 0.035 to 0.06 ml. of serum,[2] and the ascorbic acid in the white blood cells and platelets of 0.1 ml. of blood.[3]

SUMMARY

A description is given for the adaptation of the Beckman spectrophotometer to the performance of colorimetry and spectrophotometry on volumes of 0.05 ml. or less. Illustrative data and absorption curves are included. Thus adapted, the measurements on volumes of this order of magnitude are as rapid and convenient and very nearly as precise as with larger volumes.

[1] Lowry, O. H., Lopez, J. A., and Bessey, O. A., J. Biol. Chem., 160, 609 (1945).
[2] Bessey, O. A., Lowry, O. H., and Brock, M. J., unpublished data.
[3] Lowry, O. H., Bessey, O. A., and Brock, M. J., unpublished data.

A SPECTROPHOTOMETRIC STUDY OF THE COMPETITION OF METHEMOGLOBIN AND CYTOCHROME OXIDASE FOR CYANIDE IN VITRO

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Although the efficacy of induced methemoglobinemia in cyanide poisoning has been demonstrated both therapeutically and prophylactically (1),[1] the tissue reactions involved in the protection of animals from the lethal effects of the cyanide ion by methemoglobin are only imperfectly understood. Presumably, cyanide interferes with cellular respiration by forming a slightly dissociable complex with cytochrome oxidase (2–4). Kobert (5) first showed that methemoglobin combines with cyanide to form cyanmethemoglobin. The often reported antidotal action of methemoglobin-forming substances in cyanide poisoning may then be regarded as a competitive reaction between methemoglobin and cytochrome oxidase for cyanide ion. It is the purpose of the present work to determine whether this competition may be demonstrated *in vitro* and to obtain some idea of the comparative dissociabilities of cyanmethemoglobin and cytochrome oxidase-cyanide complex.

EXPERIMENTAL

A spectrophotometric technique based upon the rate of oxidation of reduced cytochrome c was used for the measurement of cytochrome oxidase activity (6, 7). Since reduced cytochrome c has a sharp absorption peak at 5500 A, the rate of oxidation can be measured by determining the change in absorption of monochromatic light at that wave-length.

Ideally, the competition of cytochrome oxidase and methemoglobin should be studied in a system in which the various components are present in known concentrations. In the present work, practically pure crystalline methemoglobin and a purified preparation of cytochrome c were used. No purified preparation of cytochrome oxidase was available. A very dilute homogenate of brain was used as the source of cytochrome oxidase.

Adult male rats, weighing about 300 gm., were decapitated and the brains quickly removed. In each experiment, the brain was rapidly weighed on a torsion balance, transferred to a Waring blendor, and enough iced distilled water added to yield a 2 per cent homogenate. The tissue was homogenized

[1] Jandorf, B. J., and Bodansky, O., *J. Ind. Hyg. and Toxicol.*, in press.

for 2 minutes, after which it was strained through several layers of surgical gauze to remove gross particles.   This stock homogenate was kept refrigerated at all times.   For any experiment, 5.0 ml. of the stock homogenate were pipetted into a tube.   Water or a solution of sodium cyanide was added to a final volume of 10.0 ml.; 0.04 or 0.05 ml. of this diluted homogenate was used in each run.   The cyanide stock solution prepared from sodium cyanide was made up in a concentration of 0.01 M and checked for cyanide content at various intervals by silver chloride titration.

Cytochrome oxidase activity was measured in the Beckman spectrophotometer at 5500 A, and a nominal slit width of 1.9 mμ.   At this wavelength, reduced cytochrome has a sharp absorption maximum.   As the reduced cytochrome is oxidized, the density decreases; the rate of decrease is a measure of the oxidase activity.   Oxidized cytochrome itself shows some absorption at 5500 A, so that the observed values really measure the decrease in density due to the disappearance of reduced cytochrome as well as a slight increase due to the accumulation of oxidized cytochrome.   If the initial concentration of cytochrome is known, as well as the absorption coefficients of reduced and oxidized cytochrome, it is possible from the observed values to calculate the concentrations of either component at any time.   The fundamental equations have been derived by Haas, Horecker, and Hogness (6), and by Altschul, Abrams, and Hogness (7), and were used in the present paper to calculate the rate of disappearance of the reduced cytochrome.   The reaction velocities are expressed in terms of the monomolecular reaction constants.

Cytochrome c was prepared from beef heart, according to the method of Keilin and Hartree (8); crystalline methemoglobin, used in many of the experiments to be described, was prepared from guinea pig blood, according to the method of Warburg and Reid (9).   From one sample of blood (approximately 30 ml.), two harvests of crystals were obtained.   The first assayed 99.2 per cent methemoglobin of the total pigment, the second 95.0 per cent methemoglobin of the total pigment.

For any day's experiments, a solution containing cytochrome c and a concentration of phosphate buffer that would give a concentration of 0.01 M and a pH of 7.4 in the final reaction mixture was used.   The cytochrome c was completely reduced with several crystals of dry sodium hydrosulfite, the excess hydrosulfite oxidized by aeration, and enough water added to give a volume of 2.95 or 2.96 ml.   The density was then read at 5500 A, after which 0.05 or 0.04 ml., respectively, of the diluted homogenate was added; the contents of the vessel were mixed and timed readings taken, beginning 1 minute after the addition of the enzyme and continuing at 30 second intervals for from 3 to 8 minutes.   Correction was made for the slight initial increase in density due to the turbidity of the enzyme source by measuring the turbidity of the homogenate in distilled water.

In the experiments in which the effect of methemoglobin on the effect of cyanide inhibition was studied, a methemoglobin solution was substituted for an equal volume of water. All final volumes were, therefore, maintained at 3.0 ml. Methemoglobin itself absorbs at 5500 A, so that, in those experiments in which it was used, the observed densities were corrected before the changes in cytochrome c concentration were calculated. In several experiments, preformed cyanmethemoglobin was used. This was prepared by reaction of methemoglobin and cyanide in amounts equivalent to those present in the final reaction mixture to which these substances were added separately. All cyanide concentrations indicated in the text are final concentrations in the reaction mixtures.

Since the Beckman spectrophotometer has no mechanism for keeping the reaction vessels at constant temperature, temperatures of the reaction mixture were recorded at the end of each determination. Within any series of experiments on the same day the variation was never greater than $1.5°$.

Results

Fig. 1 shows the typical effects of cyanide ion on the rate of oxidation of cytochrome c by cytochrome oxidase, as well as the effect of methemoglobin on the activity of the oxidase partially inhibited by cyanide. The lowest curve shows that a concentration of 3.26×10^{-6} M cyanide decreased the reaction velocity constant 85 per cent. The presence of 1.32×10^{-5} M methemoglobin (calculated on the basis of a molecular weight of 17,000 for methemoglobin) in the cyanide-inhibited system did not affect the rates for the first 2 minutes of the reaction; thereafter, the rate increased so that the inhibition, as judged by the reaction velocity constant, was 77 per cent of the activity of the control system. This indicated that methemoglobin reversed cyanide inhibition.

Fig. 1 also shows that an amount of cyanmethemoglobin present, equivalent to 1.32×10^{-5} M methemoglobin, had no initial effect on the activity of the oxidase. However, after 2 minutes, the rate of oxidation began to decrease; the final rate of oxidation represented a 79 per cent inhibition of the control rate.

Table I·shows that 1.32×10^{-5} M methemoglobin restored a cyanide-inhibited system from 24 to 35 per cent of the original activity, whereas 2.64×10^{-5} M restored it to 50 per cent and 3.96×10^{-5} M to 77 per cent of the original activity. It was not possible to study higher concentrations because of increased density of the solutions and the limitations of the instrument. Control experiments carried out simultaneously showed that the corresponding amounts of methemoglobin did not affect the rates of oxidation of cytochrome c by cytochrome oxidase in the absence of cyanide.

In the experiment described in Table I, the oxidase content and cyanide concentrations were kept constant and the methemoglobin concentration

varied. In the series of experiments shown in Fig. 2, the oxidase and methemoglobin levels were maintained constant and the cyanide concentrations were varied. Throughout the range of cyanide concentrations between approximately 5×10^{-5} M and 1×10^{-9} M, 1.32×10^{-5} M methemoglobin produced the same degree of reversal of inhibition, approximately
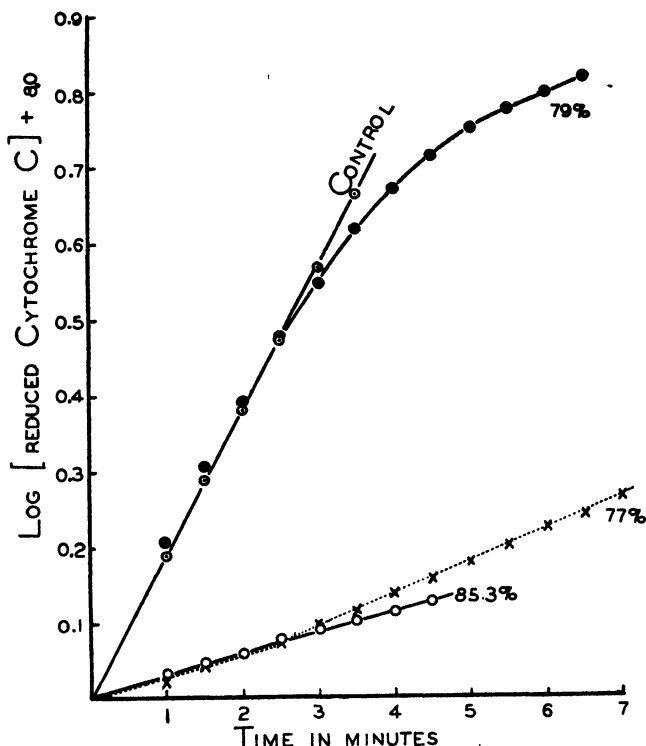
FIG. 1. The effect of methemoglobin, cyanide, and cyanmethemoglobin on brain cytochrome oxidase activity. The final reaction mixture had a volume of 3.0 ml. and contained 0.05 ml. of 1 per cent brain homogenate, 3.75×10^{-5} M cytochrome, and 0.01 M phosphate buffer, pH 7.4. ⊙ shows the reaction without any additions; O, the effect of 3.26×10^{-6} M cyanide; ✕, the effect of 1.32×10^{-5} M methemoglobin in the presence of 3.26×10^{-6} M cyanide; ● shows the effect of 1.32×10^{-5} M preformed cyanmethemoglobin on the reaction in the absence of added cyanide. The figures in per cent represent the degree of inhibition.

10 per cent of the original activity. In other words, in the presence of this amount of methemoglobin, approximately 5 times as much cyanide was required to produce a given degree of inhibition as in the absence of methemoglobin. Thus, 50 per cent inhibition was produced by 2×10^{-8} M cyanide while, in the presence of 1.32×10^{-5} M methemoglobin solution, the same inhibition was produced by 8.9×10^{-8} M cyanide.
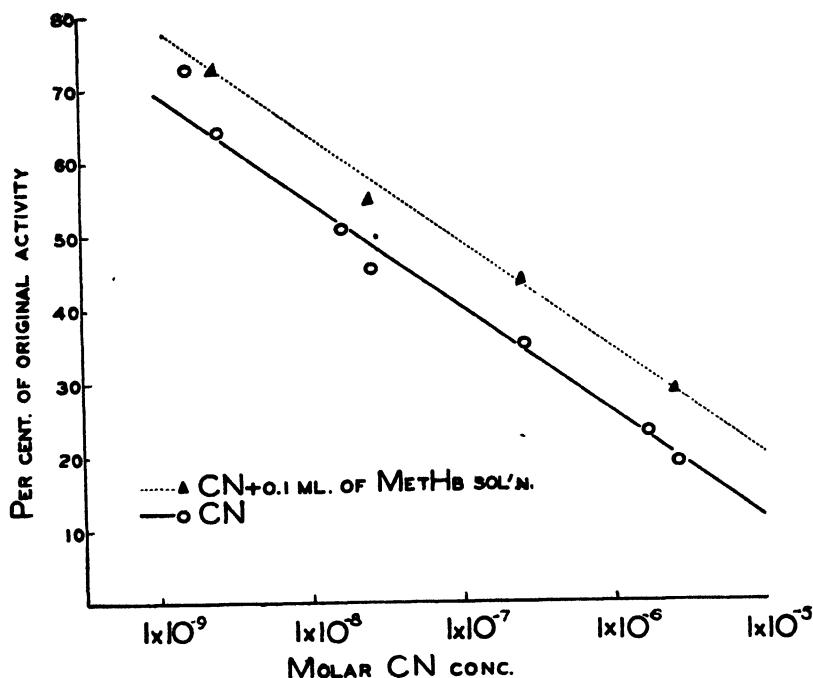
FIG. 2. The effect of varying amounts of cyanide on the activity of brain cytochrome oxidase in the presence and absence of methemoglobin. The final reaction mixture had a volume of 3.0 ml. and contained 0.04 ml. of 1 per cent brain homogenate, 2.305×10^{-5} M cytochrome, 0.01 M phosphate buffer, pH 7.4. O shows the effect of varying concentrations of cyanide; ▲, the effect of the same concentrations of cyanide in the presence of 1.32×10^{-5} M methemoglobin.

TABLE I

Effect of Varying Amounts of Methemoglobin on Cyanide Inhibition of Brain Cytochrome Oxidase Activity

The final reaction mixture had a volume of 3.0 ml. and contained 0.05 ml. of a 1 per cent brain homogenate (100 γ dry weight of tissue), 0.01 M phosphate buffer pH 7.4, and 2.69×10^{-5} M cytochrome.

Composition of reaction mixture	Reaction velocity	
	K^*	per cent
Cytochrome c + brain homogenate..........................	0.240	100
" " + " " $+ 1.13 \times 10^{-6}$ M cyanide....	0.057	24
" " + " " + cyanide $+ 1.32 \times 10^{-5}$ M methemoglobin...	0.085	35
Cytochrome c + brain homogenate + cyanide $+ 2.64 \times 10^{-5}$ M methemoglobin...	0.120	50
Cytochrome c + brain homogenate + cyanide $+ 3.96 \times 10^{-5}$ M methemoglobin...	0.185	77

* Monomolecular reaction constant calculated as in Fig. 1 after it had attained a constant maximal value.

In none of the experiments described was more than 0.05 ml. of a 1 per cent brain homogenate used as a source of enzyme. This quantity in no case corresponded to more than 120 γ of dry tissue, of which only a small part is cytochrome oxidase. This conclusion is based on the observation that such homogenates have been shown to contain varying quantities of numerous enzymes found in animal tissues. Since no pure cytochrome oxidase preparations were available from which to make molecular weight calculations, it is very difficult to know precisely how much oxidase competes with a known amount of methemoglobin for available cyanide.

However, an estimate of the competition between methemoglobin and cytochrome oxidase for the cyanide ion may be obtained in the following way. On the assumptions that in the incompletely inhibited enzyme system practically all of the cyanide ion is combined, that the concentration of the free cyanide ion is negligible, and that cyanide combines mole for mole with the oxidase iron, the total amount of oxidase in terms of moles of iron may be estimated from the cyanide concentration. For example, at 1×10^{-6} M cyanide, 76 per cent of the oxidase is inactivated; the order of magnitude of oxidase iron would be $100/76 \times 1 \times 10^{-6}$, or approximately 1.3×10^{-6} M. In the experiments recorded in Table I, the methemoglobin concentrations ranged from 1.32 to 3.96×10^{-5} M methemoglobin. At a methemoglobin-cytochrome oxidase ratio of 10:1, 11 per cent of the original activity was restored. At ratios of 20:1 and 30:1, 26 to 49 per cent of the original activities were restored. These results suggest that the cytochrome oxidase-cyanide complex is less dissociable than the cyanmethemoglobin. Moreover, as has been shown in this work (Fig. 1), cyanmethemoglobin, added to a cytochrome-cytochrome oxidase system, apparently dissociates sufficiently so that the resultant cyanide ions can inhibit cytochrome oxidase activity.

In vivo, when methemoglobinemia is induced, either prophylactically or therapeutically, to counteract the effects of cyanide ion, the latter may be considered to participate in the three following reactions:

$$CN^- + \text{cytochrome oxidase} \rightleftarrows \text{cytochrome oxidase cyanide} \qquad (1)$$

$$CN^- + \text{methemoglobin} \rightleftarrows \text{cyanmethemoglobin} \qquad (2)$$

$$CN^- + \text{S compounds} \rightleftarrows CNS^- \qquad (3)$$
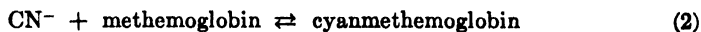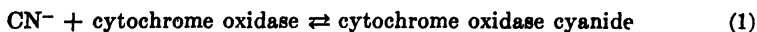
When methemoglobinemia is induced prophylactically (*i.e.* before cyanide is administered), Reaction 2 takes place in the blood stream and the amount of cyanide ion reaching the tissues is greatly decreased. When methemoglobinemia is induced after administration of cyanide and a therapeutic result is obtained, it may be assumed that methemoglobin has combined

with enough cyanide to cause Reaction 1 to shift to the left and liberate sufficient free cytochrome oxidase to permit tissue respiration to be resumed. Reaction 3 represents a pathway for the conversion of free cyanide ion to the non-toxic thiocyanate. It has been shown that administered cyanide is excreted almost completely in the urine as thiocyanate (10).

SUMMARY

1. Experiments have been performed on the *in vitro* competition between methemoglobin and cytochrome oxidase in brain homogenates for cyanide ion.

2. It is shown that methemoglobin can reverse the cyanide inhibition of cytochrome oxidase activity. The extent of the reversal was studied at varying concentrations of methemoglobin and cyanide. It is shown that in a suitable *in vitro* system preformed cyanmethemoglobin dissociates to give sufficient free cyanide ion to combine with cytochrome oxidase.

3. The *in vivo* implications of the above observations are discussed briefly.

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OBSERVATIONS ON VITAMIN B_c CONJUGASE FROM HOG KIDNEY

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Some time ago we called attention to the wide-spread occurrence in nature of an enzyme which releases vitamin B_c from a microbiologically inactive form (1). In work designed to characterize the enzyme in hog kidney through a study of this reaction it became apparent that the kinetics of the reaction were very markedly influenced by the purity of the substrate. With the isolation of vitamin B_c conjugate in crystalline form from yeast (2) the opportunity presented itself to examine the reaction with the pure conjugate as substrate. In this paper we present the results of the study along with a summary of our earlier observations on the distribution and properties of the enzyme. Our earlier observations were of necessity made with crude or partially purified substrates. In the light of later results obtained with the pure conjugate as substrate these earlier results are considered to have a more qualitative than quantitative significance.

While this work was in progress Mims, Totter, and Day (3) reported the occurrence of an enzyme in rat liver which formed a *Streptococcus faecalis*-stimulating factor from a microbiologically inactive precursor in yeast. Laskowski, Mims, and Day (4) noted the wide-spread distribution of the enzyme in the tissues of the rat, dog, hog, rabbit, chicken, and ox. They observed that chicken pancreas was an unusually rich source of the enzyme and devised a method for its purification. Their purified enzyme from chicken pancreas was found to form vitamin B_c from partially purified vitamin B_c conjugate (1, 4). Recently these workers have further purified the enzyme from chicken pancreas and studied its kinetics on a partially purified substrate (5). In the course of our study we have found some differences in the behavior of the enzyme from chicken pancreas and hog kidney toward vitamin B_c conjugate, which are reported below.

EXPERIMENTAL

Distribution of Vitamin B_c Conjugase—In the earlier phases of the work a partially purified concentrate of vitamin B_c conjugate prepared from yeast extract was used as an enzyme substrate. This concentrate contained 13 mg. of vitamin B_c per gm. of solids, as determined by assay with *Streptococcus faecalis* after a 16 hour incubation at 45° with excess of vitamin B_c conjugase.

Typical experiments with this partially purified substrate were carried out as follows: Amounts of the substrate equivalent to approximately 80 γ of vitamin B$_6$, combined as conjugate, were transferred to test-tubes and 5 cc. of 0.1 M acetate buffer at pH 4.5 or 0.1 M phosphate buffer at pH 7.0 were added. Each tissue under investigation, which was finely minced either with a Waring blendor or a Potter-Elvehjem homogenizer (6) and mixed with 3 to 4 volumes of water, was then added and the volume made up to 10 cc. with distilled water. The tubes were incubated in a water bath at 45°. At proper intervals the contents of each tube were mixed, and a 1 cc. portion withdrawn and transferred to another test-tube marked at 10 cc. This was heated 3 minutes in a boiling water bath, cooled, and diluted to 10 cc. with distilled water. After all the samples for a given

TABLE I

Vitamin B$_6$ Conjugase Activity of Various Tissues Measured at Two pH Levels

Enzyme source	Vitamin B$_6$ released per hr. per gm. tissue	
	pH 4.5	pH 7.0
	γ	γ
Chicken pancreas......................	<400	14,880
Turkey "	<1000	14,000
Rat pancreas.........................	3680	1,430
Mouse pancreas.......................	930	310
Hog liver	420	<10
Chicken liver.........................	240	<50
Hog kidney..........................	240	<13
" pancreas.........................	190	0
Guinea pig pancreas...................	58	<7
Defatted almond meal.................	50	6.5
Beef red bone marrow.................	<20	<20
" pancreas	<15	<5

experiment were collected in this way, they were assayed for vitamin B$_6$ by the method previously described (7), except that *Streptococcus faecalis* was used and the incubation time was 18 hours at 37°.

In Table I are shown the comparative potencies of various tissues investigated as sources of conjugase. The amount of tissue added to each tube during incubation with the substrate was determined by preliminary experiment so that the amount of vitamin B$_6$ released per hour was constant over a period of at least 2 hours. Potencies were evaluated in terms of the amount of vitamin B$_6$ released per hour per gm. of tissue. All incubations were carried out both at pH 4.5 and 7.0. A few tissues, including chicken pancreas and hog kidney, were incubated with the substrate at several other pH levels also. Chicken and turkey pancreas proved to be the most potent sources of conjugase. Their optimum activity was

approximately pH 7.0, whereas all other sources investigated had their optimum at approximately pH 4.5. Hog kidney was chosen as a source of conjugase for this study, since it was fairly active and available in large quantities.

Fractionation Experiments and Description of Conjugase Preparation— Hog kidney conjugase remained stable during autolysis at the natural pH of the tissue. This made the preparation of a clear water extract easier and was the basis for the preparation of a water-soluble conjugase fraction previously described (1). However, there was a great loss of activity involved in any fractionation procedure attempted.

Approximately half the conjugase activity of fresh hog kidney was lost when it was ground and desiccated with acetone. The acetone-desiccated tissue retained its remaining activity over long periods at room temperature. When water extracts of fresh kidney were dried from the frozen state, they retained their activity but soon lost it on standing at room temperature. They were quite stable in the refrigerator. One such preparation lost 74 per cent of its activity in 16 days at room temperature, but only 13 per cent in a month at refrigerator temperature. Whole kidney tissue or water extracts of kidney retained full conjugase activity for at least 5 to 6 months when maintained in the frozen state.

Less than half the conjugase activity of desiccated hog kidney could be extracted by water or phosphate buffers ranging in pH from 4.5 to 7.5. The best pH for extraction was 7.0. Physiological salt solution was better than distilled water for extraction of the enzyme but higher concentrations of sodium chloride were not as good. Clarified water extracts of fresh kidney tissue retained about three-fourths of the enzyme activity that could be demonstrated for a water suspension of the whole tissue.

The fraction precipitated from a water extract of kidney by ammonium sulfate at 40 per cent saturation contained about the same amount of conjugase activity as the fraction soluble at that concentration but insoluble at 80 per cent saturation. However, the latter fraction was about twice as active as the former in terms of its nitrogen content. The activity of both these fractions amounted to only 38 per cent of the activity of the original extract. These ammonium sulfate precipitates, or a water extract of kidney, lost at least 75 per cent of their conjugase activity when dialyzed overnight against running water. After isoelectric precipitation of a water extract of kidney at pH 4.2 to 4.5, both the soluble and insoluble fractions contained approximately equal amounts of activity but the total activity of both fractions measured separately did not equal the activity of the original extract. Both fractions were heat-labile. Acetone at 40 per cent concentration precipitated all the activity of a water extract of kidney but there was no significant purification.

In view of these findings concerning the loss of activity with fractionation, the reduced stability of purified fractions, and the lack of uniform potency of the products, the conjugase preparation used in the studies to be reported here was prepared by the following simple procedure. A fresh hog kidney was trimmed of fat, sliced, and ground in a Waring blendor with the addition of 3 cc. of water per gm. of kidney. The resulting suspension was centrifuged at 5000 R.P.M. and the supernatant solution filtered through a bed of Super-Cel. The filtrate was dispensed in test-tubes, frozen solid, and kept in a dry ice chest until used. This clarified water extract of fresh hog kidney is what is referred to throughout the balance of this paper as the conjugase preparation. It has the advantage of being highly active, it is stable when kept frozen, and it possesses a low blank. 1 cc. of this preparation, which was sufficient to release at least 2 γ of vitamin

TABLE II

Inhibitive Effect of Crude Yeast Extract on Activity of Vitamin B$_c$ Conjugase

Substrate	Amount of enzyme	Vitamin B$_c$ released per hr. per cc. enzyme
	cc.	γ
1 cc. purified vitamin B$_c$ conjugate from yeast	0.2	28.2
2 gm. Difco yeast extract	2.8	0.3
0.5 cc. purified vitamin B$_c$ conjugate from yeast plus 1 gm. Difco yeast extract	2.8	2.0

B$_c$ from a crude yeast extract under the routine digestion procedure, assayed only 0.005 γ of vitamin B$_c$ when digested alone without substrate.

Early experiments with this conjugase preparation indicated a rather broad temperature optimum at 45–48°. The studies made with partially purified substrate were carried out with an incubation temperature of 45°. Routine overnight digestions with the enzyme for the purpose of releasing a maximum of activity were carried out in an air incubator at 45°. When crystalline conjugate was used as a substrate, incubations were carried out at 37° in a constant temperature water bath unless otherwise indicated.

Inhibitors of Vitamin B$_c$ Conjugase in Yeast Extracts—In earlier studies it was observed that a given preparation of conjugase would release more vitamin B$_c$ in unit time from some substrates than from others. Crude yeast extracts were particularly refractory to the action of the enzymes, while purified concentrates were acted upon more readily. In Table II are shown the amounts of vitamin B$_c$ released from a purified vitamin B$_c$ conjugate preparation and from Difco yeast extract. 0.2 cc. of the enzyme

preparation released 28.2 γ of vitamin B$_c$ from the purified concentrate in 1 hour, while a much larger amount of conjugase released only 0.3 γ of vitamin B$_c$ from Difco yeast extract. When the substrate consisted of a mixture of these two materials, only a relatively small amount of vitamin B$_c$ was released by a large amount of the enzyme. This suggested the presence of a substance or substances in Difco yeast extract which inhibited the action of the enzyme.

Further evidence for the existence of a conjugase inhibitor in yeast extract is presented in Table III. Data are shown representing vitamin B$_c$ released per hour per cc. of conjugase preparation from substrates of different degrees of purity, ranging from a water extract of plasmolyzed brewers' yeast, which was the starting material, to crystalline vitamin B$_c$ conjugate. It will be noted that over 100 times more conjugase were required to release

TABLE III

Effect of Purity of Substrate on Action of Vitamin B$_c$ Conjugase

Sample No.	Substrate	Vitamin B$_c$ per gm. substrate*	Weight of sample	Amount of enzyme used	Vitamin B$_c$ released per hr. per cc. enzyme
		γ	*mg.*	*cc.*	γ
38843	Yeast extract	84	1000	4	1.0
90485	" Concentrate A	366	360	3.6	2.25
90515	" " B	1,250	44	0.4	9.4
80004	" " C	13,000	6	0.16	36
91965	Crystalline vitamin B$_c$ conjugate	365,000	0.11	0.004	139

* As measured by assay with *Streptococcus faecalis* after a 16 hour incubation at 45° with excess of enzyme, except Sample 91965.

1 γ of vitamin B$_c$ from yeast extract than from crystalline vitamin B$_c$ conjugate.

Experiments with Crystalline Vitamin B$_c$ Conjugate As Substrate—Most of these experiments were carried out on a smaller scale than those above. Digestion mixtures had a total volume of 1 or 2 cc. Unless otherwise indicated, the digestions were carried out in 0.05 M acetate buffer at pH 4.5.

The effect of pH on the action of conjugase from hog kidney and chicken pancreas, when crystalline conjugate was used as substrate, is shown in Fig. 1. 11.2 γ amounts of the conjugate were incubated with 6.5 mg. of hog kidney and 0.1 mg. of chicken pancreas, respectively, in 1 cc. volumes of 0.05 M buffer for 1 hour over the pH ranges shown. Acetate buffer was used below pH 4.5, phosphate buffer above. It was found that phosphate and acetate buffers gave identical results at pH 4.5.

After incubation the contents of the tubes were heated in boiling water,

diluted to 10 cc., and assayed. The optimum pH for hog kidney was 4.3 to 4.5, while for chicken pancreas it was 7.0 to 7.5. In other experiments it was found that these same pH optima also held for hog kidney and chicken pancreas when the substrates were crude yeast extract or a partially purified concentrate of vitamin B$_c$ conjugate obtained from yeast extract. The pH optimum of 7.0 to 7.5 found for chicken pancreas confirms the optimum observed by Laskowski *et al.* (4), but not the value of pH 5.4 reported by Burkholder *et al.* (8).

The result of hog kidney conjugase acting upon an excess of pure vitamin B$_c$ conjugate is indicated in Fig. 2. The response with time was linear, within the error of the microbiological assay, over a 4 hour time interval.
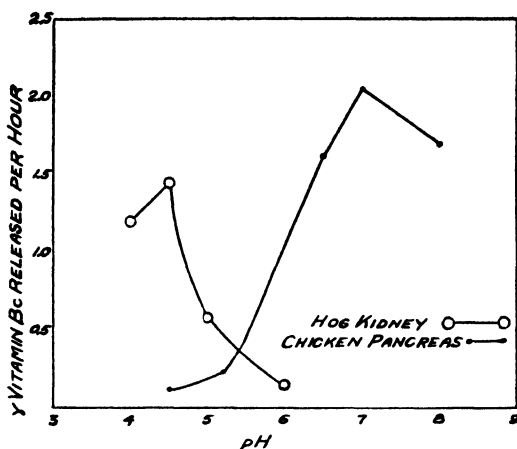
FIG. 1. Reaction velocity of hog kidney conjugase and chicken pancreas conjugase as a function of pH. Each tube for establishing the hog kidney conjugase curve contained 10 mg. of hog kidney and 112 γ of crystalline vitamin B$_c$ conjugate in 10 cc. For the chicken pancreas curve each tube contained 2 mg. of pancreas and 11.2 γ of crystalline conjugate in 1 cc. Incubation was at 37°.

Longer periods were not studied. In Fig. 3 is shown the effect of increasing concentrations of conjugase upon the amount of vitamin B$_c$ released from a constant amount of substrate. It will be noted that beyond the point where approximately 0.2 γ of vitamin B$_c$ per cc. per hour was released the rate of liberation per unit volume of enzyme solution was fairly constant.

The data in Fig. 4 indicate the velocity of the action of hog kidney conjugase as a function of substrate concentration. Increasing amounts of crystalline vitamin B$_c$ conjugate, ranging from 2.8 γ (equivalent to 1 γ of free vitamin B$_c$) to 16.8 γ per tube were incubated in the presence of 4 c.mm. of the conjugase preparation. Half maximum velocity of the reaction was reached at a concentration of approximately 4.5 γ of vitamin B$_c$ conjugate per cc. of enzyme solution.

Definition of Conjugase Unit—The results of the above experiments on the action of conjugase on a substrate of crystalline conjugate suggested the definition of a unit of conjugase activity. A unit of activity, as applied
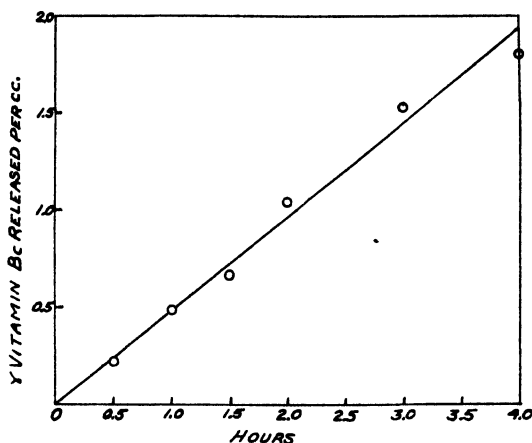
Fig. 2. Relationship of formation of vitamin B$_c$ to total reaction time. The reaction mixture contained 224 γ of crystalline vitamin B$_c$ conjugate and 40 c.mm. of hog kidney conjugase preparation in a volume of 10 cc. Incubation was at 37°.
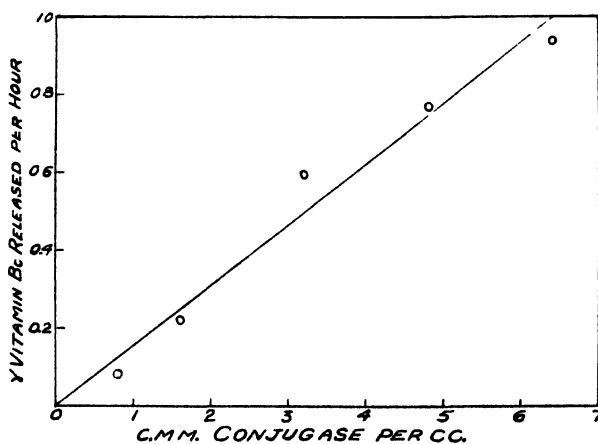
Fig. 3. Relationship of rate of formation of vitamin B$_c$ to concentration of hog kidney conjugase. The reaction mixture contained 2.8 γ of crystalline vitamin B$_c$ conjugate per cc. and was incubated at 37°.

to the conjugase present in hog kidney or other tissues containing conjugase having a pH optimum at 4.5, is that amount of enzyme which produces 1 γ of vitamin B$_c$ from crystalline conjugate in 1 hour under the following

conditions. The reaction mixture should contain an amount of crystalline vitamin B₆ conjugate equivalent to 1 γ of vitamin B₆ per cc., and a concentration of conjugase such that 0.2 to 0.8 γ of vitamin B₆ will be released when incubated 1 hour at 37° in 0.05 M buffer at pH 4.5. The conjugase preparation described above and used in these studies contained 150 to 200 of these units per cc.

Complete Enzymatic Release of Vitamin B₆ from Its Conjugate—The specific ultraviolet absorption properties of crystalline vitamin B₆ conjugate indicated that approximately 36 per cent of the conjugate molecule consisted of vitamin B₆ (2). Before digestion with conjugase pure vitamin B₆ conjugate was found to have only a negligible growth effect on *Lactobacillus*
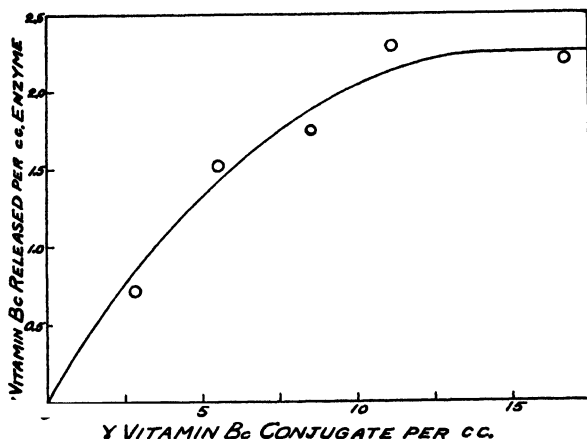
FIG. 4. Reaction velocity of hog kidney conjugase as a function of substrate concentration. Each reaction tube contained 0.4 c.mm. of hog kidney conjugase preparation per cc. and was incubated at 37°.

casei and *Streptococcus faecalis* (2). Its utilization as a growth factor by these two organisms is discussed in the following paper (9).

It was of interest therefore to determine whether enzyme treatment of the pure conjugate would release an amount of vitamin B₆ comparable to that indicated from spectrophotometric examination. A number of pure specimens were examined by both methods. The data are presented in Table IV.

Enzymatic microbiological assay of these pure preparations consisted, first, of diluting the conjugate solution to an estimated concentration (based on spectrophotometric assay) of 2 γ of vitamin B₆ per cc. To 1 cc. of this dilution were added 1 cc. of the conjugase preparation (150 to 200 units) and 5 cc. of 0.1 M acetate buffer at pH 4.5. After addition of a few drops of toluene the mixture was incubated 16 hours at 45° in an air incu-

bator, adjusted to pH 7.0 by the addition of N NaOH, diluted to 10 cc., autoclaved briefly, and assayed for vitamin B_c, as indicated previously. The spectrophotometric values reported in Table IV were determined from the extinction coefficients of crystalline vitamin B_c conjugate (2).

It will be noted that a large excess of conjugase was used in releasing vitamin B_c from these conjugate preparations, much larger, in fact, than would be calculated to be necessary. The correlation between microbiological and spectrophotometric measurements is good in all cases but one. No explanation is apparent for this failure. Although some preparations of pure conjugate have been converted completely to free vitamin

TABLE IV

Microbiological Assay of Crystalline Vitamin B_c Conjugate Preparations Following Treatment with Hog Kidney Conjugase; Comparison with Spectrophotometric Determinations

Sample No.	Vitamin B_c per cc.		
	Spectrophotometric*	Microbiological assay after enzyme treatment†	
		Lactobacillus casei	*Streptococcus faecalis*
	γ	γ	γ
515	16	13.3	15
655	8	6.0	
701	32	23	
720	17	15	
795	12.5	10.3	9.7
91965	40	38	
1272	13	13	
1291	39	38.5	37

* As estimated from the specific ultraviolet absorption properties of crystalline vitamin B_c conjugate (2).

† Incubated with hog kidney conjugase and assayed according to a previously reported method (7).

B_c with only a small excess of conjugase, others have required a huge excess to accomplish the conversion, which even then was not always complete.

DISCUSSION

It seems remarkable that of the tissues examined as sources of vitamin B_c conjugase all had pH optima at approximately 4.5, except chicken and turkey pancreas whose optima were at pH 7.0 to 7.5. Since the pH optimum for chicken pancreas was unaffected by the purity of substrate used, it seems probable that a different conjugase is present in chicken pancreas, and perhaps the pancreas of other fowl, than is found in the organs of mammals.

The demonstration of the presence of inhibitors for vitamin B$_c$ conjugase in crude yeast extracts and the fact that they are not readily eliminated during fractionation of these extracts indicate the possibilities of error in using a crude substrate to measure enzyme concentration. Since crude substrates such as yeast extracts or fractions prepared from them vary widely in their inhibitor content, the advantage of using the pure, crystalline vitamin B$_c$ conjugate as a substrate in standardizing the enzyme preparations is apparent.

Mims and Laskowski (5) have mentioned the possible occurrence of a coenzyme for vitamin B$_c$ conjugase from chicken pancreas. In view of the loss of activity following precipitation with ammonium sulfate, dialysis, and other fractionation procedures, it seems likely that there also exists in hog kidney a coenzyme or enzyme activator for conjugase from this source. A frequently noted characteristic of this enzyme reaction has been its failure to go uniformly to completion, perhaps again indicating the importance of some coenzyme or enzyme activator. However, experiments designed to demonstrate the existence of a coenzyme or enzyme activator for hog kidney conjugase have thus far yielded only negative results. Mims and Laskowski (5) have indicated that the calcium ion serves as an activator for chicken pancreas conjugase but we have been unable to activate dialyzed hog kidney conjugase with calcium ion.

SUMMARY

The distribution of vitamin B$_c$ conjugase in a number of tissues has been studied, with a partially purified preparation of vitamin B$_c$ conjugate as substrate. All the sources exhibited a pH optimum at approximately 4.5 except chicken and turkey pancreas, which had an optimum at pH 7.0 to 7.5.

The presence in yeast extract of a strong inhibitor for vitamin B$_c$ conjugase has been demonstrated. This inhibitor was gradually removed during fractionation of yeast extract in the preparation of pure conjugate.

The reaction of hog kidney conjugase has been studied with crystalline vitamin B$_c$ conjugate as substrate. A unit of conjugase activity has been defined, based on the amount of pure conjugate split by the enzyme in unit time under specific conditions.

A comparison is made of the vitamin B$_c$ content of several preparations of crystalline vitamin B$_c$ conjugate as determined spectrophotometrically and by microbiological assay following treatment with hog kidney conjugase.

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THE RESPONSE OF LACTOBACILLUS CASEI AND STREPTOCOCCUS FAECALIS TO VITAMIN B$_c$ AND VITAMIN B$_c$ CONJUGATE

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Our earlier attempts to assay natural products, such as extracts of yeast and liver, for uncombined vitamin B$_c$ by the *Lactobacillus casei* method recently described (1) led to inconsistent results. The potency values calculated for individual assay tubes increased markedly with each increase in amount of assay material added. When the turbidity readings for tubes containing these natural products were plotted against dosages, an exaggerated S-shaped curve resulted, compared to the typical parabolic curve obtained for crystalline vitamin B$_c$, or concentrates of vitamin B$_c$. These irregularities in assay results were particularly noticeable in a study of hog kidney conjugase, reported in the preceding paper (2), when an attempt was made to use *Lactobacillus casei* in assaying natural materials in which the vitamin B$_c$ conjugate had been only partially converted to vitamin B$_c$ by conjugase action.

We tried to explain this difficulty on the basis of inadequacy of the assay medium. Teply and Elvehjem (3) apparently noticed a similar phenomenon and attempted to improve the assay procedure by introducing a new medium which included norit-treated peptone as a stimulatory factor. However, we were unsuccessful in modifying the medium so as to obtain assays without a drift in potency values when calculated over a major part of the standard curve. Assays in which natural products were first treated with enzyme to release the vitamin B$_c$ from its conjugate did not give this abnormal response curve, and at first we attributed this difference to the presence in these materials of an unrecognized growth stimulant for *Lactobacillus casei* whose effect was lost in the greater dilution necessary in assaying the enzyme-treated samples. Recently, when vitamin B$_c$ conjugate was isolated in crystalline form (4), an opportunity was provided for determining whether or not this abnormal response was due to a supplementary stimulating factor.

EXPERIMENTAL

The assay procedure and method of treating samples with a conjugase preparation to liberate vitamin B$_c$ from its conjugate were those recently described (1). The conjugase preparation used was a clarified water

extract of fresh hog kidney which was kept frozen in test-tubes until used. When *Streptococcus faecalis* was used as the test organism, the medium was the same but the incubation period was reduced to 18 hours.

Typical dosage-response curves for crystalline vitamin B$_c$, crystalline vitamin B$_c$ conjugate,[1] yeast extract, and liver extract are shown in Fig. 1.
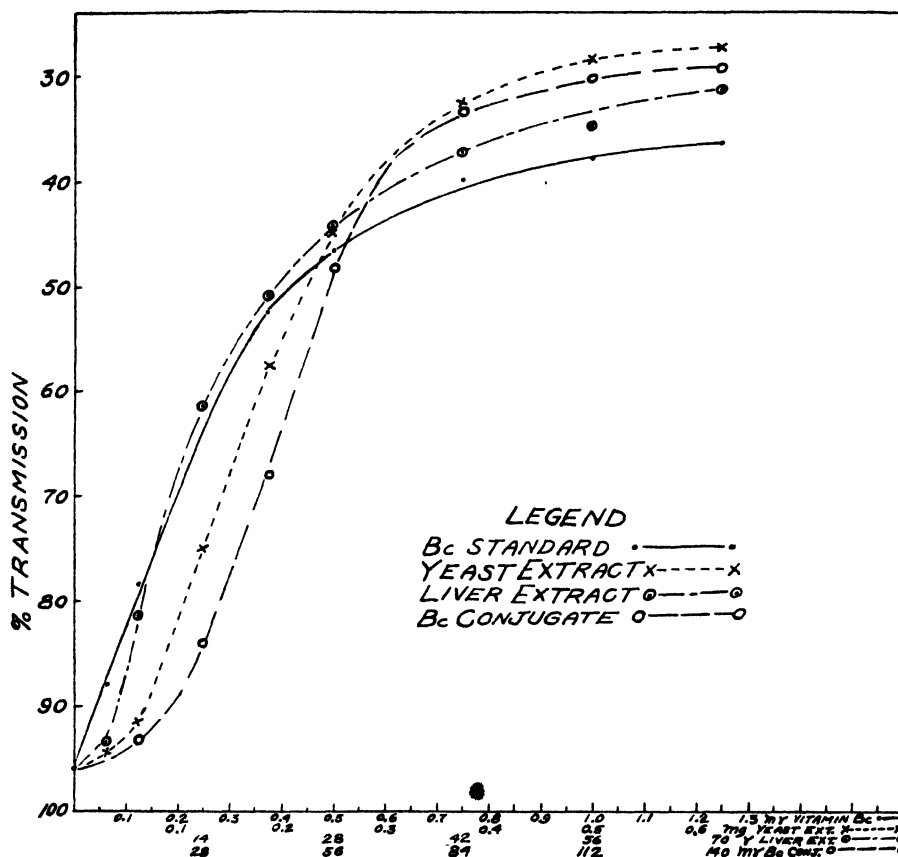
FIG. 1. Response of *Lactobacillus casei* to materials containing vitamin B$_c$ conjugate before enzyme treatment.

These curves indicate the abnormal response of *Lactobacillus casei* to crude materials containing a mixture of free and conjugated vitamin B$_c$, and to crystalline vitamin B$_c$ conjugate. Divergence from the standard curve is particularly striking in the case of both yeast extract and crystalline vitamin B$_c$ conjugate. This is illustrated in the first part of Table I, where po-

[1] Kindly supplied by Dr. J. J. Pfiffner.

tency values calculated from individual tubes containing graded amounts of yeast extract are shown. They vary over a range of more than 300 per cent, while the reading for the last tube was too high to be calculated from the standard curve.

The three sources of vitamin B_c conjugate described above were then incubated 16 hours at 45° with vitamin B_c conjugase in excess and assayed with *Lactobacillus casei*. The average dosage-response curve for all three samples and the standard is shown in Fig. 2. It will be seen how the wide divergence of response from the standard curve has disappeared following enzyme treatment. This is also shown in the second part of Table I, where potency values calculated for individual tubes containing yeast

TABLE I

Comparison of Vitamin B_c Assay Values for Yeast Extract, Calculated from Individual Assay Tubes before and after Enzyme Treatment

Before enzyme treatment			After enzyme treatment		
Amount of sample per tube	Evelyn readings	Calculated vitamin B_c per gm.	Amount of sample per tube	Evelyn readings	Calculated vitamin B_c per gm.
γ		γ	γ		γ
31.2	95	0.64	3	72	50
62.5	92	0.64	6	59.5	53.3
125	75.5	1.12	9	53	53.3
187	58	1.54	12	49	50.8
250	45.5	2.24	15	44	53.4
375	33		18	42	54
			24	39	50
Average.......					52

extract after enzyme treatment are shown. There is less than 10 per cent variation among the seven individual values.

In Fig. 3 is shown a dosage-response curve for a mixture of free and conjugated vitamin B_c compared to the standard curve for vitamin B_c, *Streptococcus faecalis* being used as the assay organism. In this case the points representing the response of this organism to the mixture of free and conjugated vitamin B_c fall almost exactly on the standard curve.

DISCUSSION

It is generally considered that good matching of the dosage-response curves for a crude assay sample and a pure standard is the most reliable criterion of a good microbiological assay. This condition also may be described as freedom from drift in potency values calculated for individual
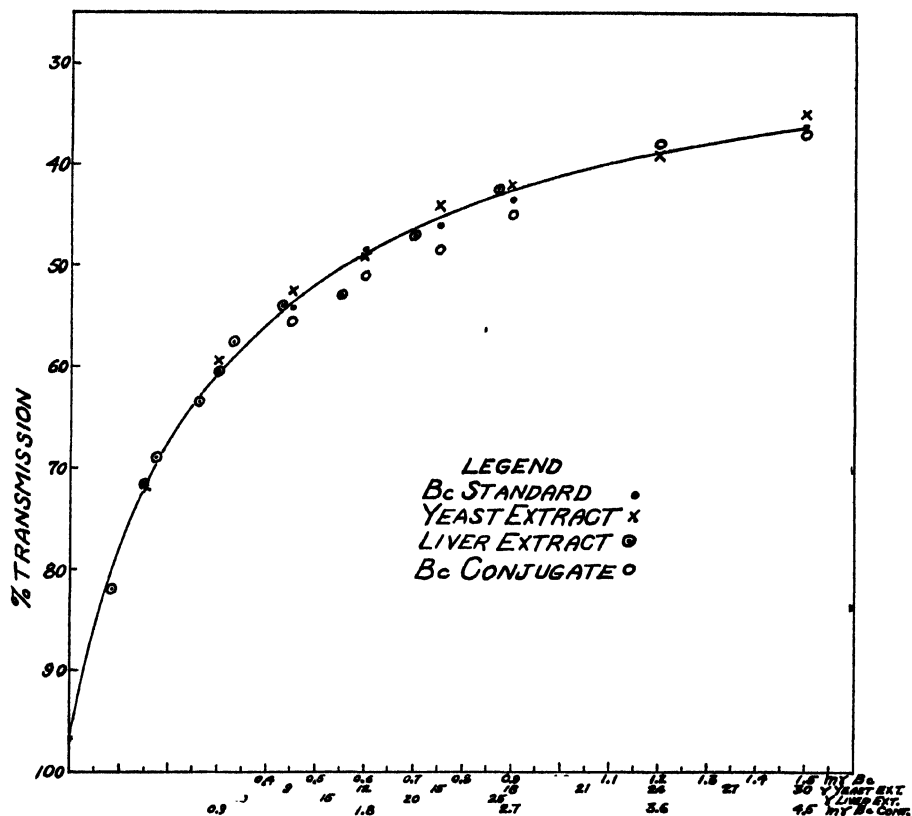
Fig. 2. Response of *Lactobacillus casei* to materials containing vitamin B$_c$ conjugate after enzyme treatment.
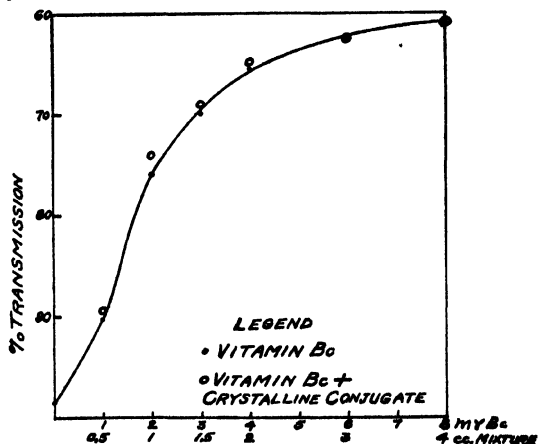
Fig. 3. Response of *Streptococcus faecalis* to a mixture of free and conjugated vitamin B$_c$. The mixture contained 1.59 millimicrograms of crystalline vitamin B$_c$ and 177 millimicrograms of crystalline vitamin B$_c$ conjugate per cc.

tubes in an assay. It is evident that this condition is not met when samples containing both free and conjugated vitamin B$_c$ are assayed with *Lactobacillus casei* prior to treatment with vitamin B$_c$ conjugase. This is also true when a sample containing crystalline vitamin B$_c$ conjugate is assayed without enzyme treatment. Calculated potency values for individual assay tubes show an extreme drift and it is impossible to get a satisfactory assay for the true amount of free vitamin B$_c$ present. This is not the case when *Streptococcus faecalis* is used to assay a mixture of free and conjugated vitamin B$_c$. The presence of conjugate does not cause an undue drift in assay values for individual tubes. Thus a mixture of free and conjugated vitamin B$_c$ may be much more satisfactorily assayed for free vitamin B$_c$ with *Streptococcus faecalis* than with *Lactobacillus casei*.

This criticism of the *Lactobacillus casei* assay does not apply if the vitamin B$_c$ present as a conjugate has been entirely converted to the free form by adequate enzyme treatment prior to the assay procedure. The close matching of response curves for unknown and standard following treatment of the former with an enzyme preparation indicates that the 40 hour assay procedure used in the experiments reported here is equal in accuracy to 72 hour procedures employing acid titration.

The S-shaped response curves obtained when samples containing vitamin B$_c$ conjugate are assayed with *Lactobacillus casei* prior to enzyme treatment are unexplained. One possible explanation may be that this organism produces an enzyme which splits the conjugate, thus freeing vitamin B$_c$ which in turn stimulates the growth of the organism. This phenomenon would be more pronounced in the tubes containing larger amounts of the conjugate, thus leading to the S-shaped response curve.

SUMMARY

The abnormal growth response of *Lactobacillus casei* obtained with crude substances containing vitamin B$_c$ conjugate is also obtained with crystalline vitamin B$_c$ conjugate. This abnormal response is entirely eliminated by treatment of crude substances with a conjugase preparation which converts the vitamin B$_c$ present to the free form.

Streptococcus faecalis does not display this abnormal response when used to assay free vitamin B$_c$ in the presence of vitamin B$_c$ conjugate.

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STUDIES ON THE NATURE OF CARBOHYDRATE ACTIVATORS OF POTATO PHOSPHORYLASE*

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Potato phosphorylase catalyzes the formation of a linear polysaccharide from glucose-1-phosphate (1). The reaction is dependent upon the presence of some types of polysaccharide in relatively small amounts. Such polysaccharides are believed to function as a source of short, linear chains onto which glucose from the substrate is added repeatedly in 1,4-α-glucosidic linkages by phosphorylase, thus forming long unbranched chains (2). The terms primer (3, 4), catalyst (5), and activator (2, 6) have been used to designate them.

In attempts to learn the nature of potato phosphorylase activators it has been found that glucosidic linkages characteristic of glycogen (*i.e.* 1,6-α-glucosidic) are not required (2, 7). Partial acid hydrolysis of amylose (Fraction A, Schoch) and several natural starches markedly increase the activating power (6, 7). Estimations based on reactions of the activator fractions with iodine indicate that activation can be produced by linear dextrins containing not more than 7 or 8 glucose units per molecule (7). Data reported in a symposium (2), since the work to be presented here was concluded, show that β-amylase decreases the activating power of amylose and of amylopectin (Fraction B, Schoch), and that controlled acid hydrolysis of Schardinger dextrins produces activators of potato phosphorylase.

The purpose of the present study was to secure additional evidence concerning the structure and size of carbohydrates capable of activating potato phosphorylase.

EXPERIMENTAL

The potato phosphorylase and glucose-1-phosphate were prepared in accordance with the methods described by Hidy and Day (7). A modification (7) of the Green and Stumpf (5) procedure for the determination of phosphorylase activity was used.

Effect of Different Carbohydrates on Activity of Potato Phosphorylase—A

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number of different carbohydrates were tested with respect to their ability to activate potato phosphorylase. Each sample, except the amylose, was prepared by dispersing it in water, with heating if necessary. The amylose samples were dispersed in 1.7 N potassium hydroxide and quickly neutralized with hydrochloric acid. The same enzyme preparation was used to make all the determinations. ·

The results are given in Table I. They show that amylopectin had the greatest activating ability. Our evidence indicates that its activity cannot be raised appreciably by acid hydrolysis (7). This is in agreement with the findings of Cori et al. (2). That unhydrolyzed amylopectin is substantially different from liver glycogen is shown in Table I. It is also demonstrated by the results of partial acid hydrolysis on the ability of these materials to activate phosphorylase from potatoes and from muscle (2).

In respect to structure, the results with inulin, dextran, and the polysaccharide of pneumococcus type III and of *Phytomonas tumefaciens* are of interest. Because inulin is a fructosan it is not surprising that it had no activity. Unhydrolyzed dextran had less than 30 per cent of the activity of soluble potato starch. Since it appears to be a 1,6-α-glucosidic structure (8), this may be the basis for the relatively low activity. The activity of the polysaccharide of type III pneumococcus was similarly low. Its structure has been reported to involve glycosidic linkages which alternate between positions 1,3 and 1,4 (9). Also, the glucuronosidic linkages have the β configuration. The polysaccharide produced by *Phytomonas tumefaciens* had no activity. The configuration of the constituent d-glucose units appears to be of the β type (10).

Only one of the five dextrins reported here had relatively high activating ability. This dextrin was prepared[1] by treatment of a corn-starch slurry with hydrochloric acid to make a highly soluble starch. This was followed by a malting process which, on the basis of copper-reducing values, was much less complete than in the case of starch not treated with acid previous to malting. Methanol was added to precipitate the limit dextrin from the soluble malted material. Each molecule of this dextrin contained an average of approximately 15 glucose units.[2] Although the calculated average number of glucose units per molecule of malted corn-starch is almost identical (17 units), it had no activity. Carbohydrates tested which had 3 hexose units or less had no activity. Thus structure, as well as molecular size, is important for activating ability even in materials which have a relatively high concentration of terminal end-groups.

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[1] Personal communication from Dr. D. P. Langlois.

[2] Estimated on the basis of the Hixon R_{Cu} value of this dextrin,

$$\frac{R_{Cu} \text{ of maltose (1900)} \times 2}{R_{Cu} \text{ of sample (259)}} = 15 \text{ glucose units}$$

TABLE I

Effect of Different Carbohydrates on Activity of Potato Phosphorylase

Each test mixture consisted of 1.0 ml. of enzyme solution ((7), p. 277), 0.5 ml. of 1.0 M citrate buffer of pH 6.2, 1.0 ml. of test carbohydrate, and 1.0 ml. of 0.1 M glucose-1-phosphate.

Carbohydrate	Source*	Amount added to reaction mixture	Activity†
		mg.	
Soluble potato starch, fat-free	Merck	20	140
" " " "	"	5	100
Amylose from corn-starch	Schoch	5	50
" " "	Kerr	5	50
Amylopectin from corn-starch	Schoch	5	116
Glycogen from liver	Eastman	20	40
" " corn purified with NaOH	Morris	20	0
" " " " " acetic acid	"	20	0
"Whole corn polysaccharide" alcohol pptd. from aqueous extract	"	20	39
Dextran from *Leuconostoc mesenteroides*	Hassid	20	40
Polysaccharide produced by *Phytomonas tumefaciens*	Hodgson and Peterson	20	0
Polysaccharide produced by type III pneumococcus	Goebel	20	40
Inulin	Eastman	20	0
Corn-starch oxidized with periodic acid	Hidy	20	0
Limit dextrin from malted HCl-treated corn-starch	Langlois	20	97
Limit dextrin from malted corn-starch	"	20	0
" " " " corn syrup insoluble in 85% ethanol	"	20	30
Limit dextrin from malted corn syrup insoluble in 75% ethanol	"	20	17
Limit dextrin from malted waxy maize starch	"	20	17
Raffinose	Eastman	20	0
Melezitose	"	20	0
Melibiose	"	20	0
Cellobiose isolated from cotton	Student's preparation	20	0
Gentiobiose synthesized	Proehl	20	0

* Grateful acknowledgment is made to the following persons for supplying some of the materials used: T. J. Schoch and R. W. Kerr, Corn Products Refining Company; D. L. Morris, Mead Johnson and Company; W. Z. Hassid, University of California; R. Hodgson and W. H. Peterson, University of Wisconsin; W. F. Goebel, The Rockefeller Institute for Medical Research; P. H. Hidy, Baylor University; and D. P. Langlois, A. E. Staley Manufacturing Company.

† All results are expressed as per cent of the value for 5 mg. of soluble corn-starch, used as the standard. With this amount of activator 0.375 mg. (corrected) of inorganic phosphorus is liberated in 10 minutes under the conditions prescribed.

Dialyzability of Phosphorylase Activators—Amylose (Schoch) was hydrolyzed by hydrochloric acid, with the method previously described (7), until the activating ability was maximum. This coincided approximately with the achromic point. It was then neutralized with potassium hydroxide and dialyzed at 4° against distilled water in Visking tubing. More than one-half of the total activating power was concentrated in the dialysate. Thus both dialyzable and non-dialyzable activators are formed by acid hydrolysis of amylose.

Effect of Alkali on Ability of Amylose to Activate Potato Phosphorylase—Amylose (Schoch) was kept dispersed in 2 N sodium hydroxide for 56 hours at 24° and then neutralized with hydrochloric acid. The activating power was not changed by the prolonged treatment with alkali.

Effect of β-Amylase on Ability of Amylose and of Amylopectin to Activate Potato Phosphorylase—The method of Ballou and Luck (11) was used to prepare the β-amylase. To carry out the enzymatic reactions 500 mg. of amylose or amylopectin, as required, were dispersed in about 30 ml. of 1.8 N sodium hydroxide and immediately neutralized with dilute hydrochloric acid. To this were added 50 ml. of 0.1 M acetate buffer of pH 4.8. The volume was adjusted to 98 ml. and the mixture was kept at 30°. Finally 2 mg. of β-amylase in 2 ml. of water were added. At specified intervals the desired aliquots were removed for determinations of phosphorylase-activating ability and reducing value, respectively; but previous to these determinations the aliquots were placed in a boiling water bath for 10 minutes to inactivate the β-amylase. The ferricyanide method of Blish and Sandstedt (12) was used to determine the reducing values.

The results are given in Table II. It is evident that the power of both polysaccharides to activate phosphorylase decreases progressively with the action of β-amylase. In contrast to the effect of acid hydrolysis there was at no time an increase in the ability to activate phosphorylase. By the time maximum hydrolysis had occurred, as indicated by the ferricyanide-reducing values, the ability of the residual limit dextrins to activate phosphorylase was practically nil. The results may be interpreted as evidence that the phosphorylase-activating ability of a carbohydrate is directly related to the concentration of non-maltosidic terminal glucose units, and that the chain length is a determinant of activating power. The latter is indicated by the evidence that β-amylase-treated amylopectin has just as high concentration of terminal glucose units as the untreated polysaccharide (13); yet the activating ability is reduced. Since the outer branches of amylopectin molecules probably do not contain more than 6 or 7 glucose units per linear branch (13), it may be concluded that the optimum chain length for phosphorylase-activating ability must be as great as these branches; otherwise the action of β-amylose would not promptly reduce that ability.

Effect of Acid Hydrolysis on Ability of Schardinger Dextrins to Activate Potato Phosphorylase—Two facts indicated that an activator of potato phosphorylase need not contain more than 7 or 8 glucose units per molecule. They were (*a*) ability of achrodextrins to activate the enzyme (7) and (*b*) the ability of activator material to dialyze. It was therefore logical to consider the activating ability of simple dextrins of known composition. The Schardinger dextrins were a logical choice because the structures of both the α and β forms seem to be well established and the number of glucose units per molecule is 6 and 7 respectively.

Owing to the absence of terminal non-maltosidic glucose units in these cycloamyloses, cleavage of the molecules by mild acid treatment was

TABLE II

Effect of β-Amylase on Ability of Amylose and of Amylopectin to Activate Potato Phosphorylase

The composition of the test mixture is the same as in Table I.

Time	Amylose		Amylopectin	
	Reduction*	Activity (P liberated in 10 min.)	Reduction*	Activity (P liberated in 10 min.)
min.	*per cent*	*mg.*	*per cent*	*mg.*
0	0	0.19	0	0.44
15			37	0.31
30	38	0.15	49	0.15
45			54	0.12
60	60	0.12	58	0.10
90	74	0.12	60	0.10
120	82	0.11	61	0.08
180	90	0.11	62	0.07
240	93	0.09		
300	94	0.09		
360	94	0.09		

* Expressed as per cent maltose.

employed with the expectation that short chain amyloses (linear) would be formed.

Several different hydrolytic conditions were studied. The results represented in Fig. 1 were obtained as follows: 0.5 gm. of dextrin was dissolved in 7.5 N hydrochloric acid. The volume was adjusted to 10.0 ml. The temperature was 24°. At specified intervals 1.0 ml. aliquots were removed, neutralized with 1.7 N sodium hydroxide, and the volume adjusted to 10.0 ml. Aliquots of these diluted neutral solutions were used within a few hours for determinations of phosphorylase activity and ferricyanide-reducing abilities.

As shown in Fig. 1, when either the α- or β-Schardinger dextrin is used as

the only added activator of phosphorylase, the rate of phosphate liberation is significantly decreased, as compared with blanks to which no source of activator was added. The magnitude of the effect was in proportion to the amount of the cycloamylose added. Since there was enough residual activator in the purified glucose-1-phosphate and potato phosphorylase to promote appreciable polysaccharide synthesis (7), the effect of Schardinger dextrins may be regarded as a form of competitive inhibition, as interpreted by Green and Stumpf (5), who also noted the inhibiting effect.

As shown in Fig. 1, the inhibiting effect of both dextrins was rapidly abolished by hydrolysis with acid and they became activators of the phosphorylase. After the maximum activating ability was reached, the effect gradually diminished to zero.
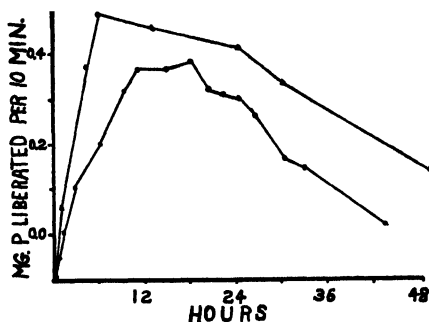
FIG. 1. Effect of hydrolysis by 7.5 N HCl on the potato phosphorylase-activating power of α- and β-Schardinger dextrins. Temperature 24°. The abscissa represents the length of hydrolysis; the ordinate, the rate of phosphorus liberation in the presence of 5 mg. of hydrolyzed dextrin. Lower curve, α-Schardinger dextrin; upper curve, β-Schardinger dextrin.

The activity of the hydrolyzed β-dextrin was approximately 30 per cent greater than the α-dextrin hydrolyzed to a comparable degree, as determined by ferricyanide reduction. This indicates that the hydrolytic products from the former dextrin have greater activating power than those from the latter. Presumably the average chain length of the β-dextrin products is 1 glucose unit greater than the products from the α-dextrin. Therefore, linear dextrins containing 6 or less glucose units may not be as capable of activating potato phosphorylase as those containing 7, or perhaps more, glucose units. Due to the inhibiting effect of the unhydrolyzed dextrins, fully satisfactory comparison of the activating ability of the hydrolytic products cannot be made until the latter have been isolated in pure form.

Special significance should be attached to the evidence that both of the Schardinger dextrins competitively inhibit polysaccharide synthesis, whereas the opposite effect is caused by dextrins formed by acid hydrolysis

of either of these compounds. The number of glucose units in each case is of the same magnitude. According to present concepts of structure (14, 15), the only difference between inhibition and activation, in this case, is that the former effect is associated with a closed ring structure, whereas the latter is associated with an unclosed ring (helix). Therefore, on the basis of present views concerning the relation of chemical structure to biological activity (16), it might be postulated that unhydrolyzed Schardinger dextrins inhibit polysaccharide synthesis because they are so similar in configuration and dimensions to the activator groups (helical, open chain) that they are able to compete with the latter for essential reactive positions on the enzyme. Owing to the lack of terminal glucose units in the

TABLE III*

Effect of Acid Hydrolysis on Ability of Schardinger Dextrins to Activate Potato Phosphorylase

Each test mixture contained the equivalent of 5 mg. of dextrin. The hydrolysis mixture contained 1.0 gm. of dextrin per 20.0 ml. of 11 N H_2SO_4 at 24°.

Time	α-Schardinger dextrin		β-Schardinger dextrin	
	Reduction*	Activity (P liberated in 10 min.)	Reduction*	Activity (P liberated in 10 min.)
hrs.	per cent	mg.	per cent	mg.
0	0	−0.10†	0	−0.01†
10	8	0.10	8	0.15
22	14	0.14	9	0.25
36	19	0.19	17	0.33
46	25	0.21	23	0.33
76			30	0.35
98	52	0.43		
104			48	0.34

* Expressed as per cent glucose.

† The negative values indicate that the dextrins inhibited the activating ability of the polysaccharide contaminating the enzyme preparation and the glucose-1-phosphate so that the phosphorus values were lower than those of the blanks.

Schardinger dextrins, glucose units from the substrate would not be added; consequently polysaccharide synthesis would be blocked.

Hydrolysis with Sulfuric Acid—The differences between hydrochloric acid and sulfuric acid on the hydrolysis of the cycloamyloses were quite marked. When 7.5 N sulfuric acid was used, the rate of hydrolysis at 24° was exceedingly slow as compared with hydrochloric acid of equal strength. As shown in Table III, 11 N sulfuric acid was also quite slow. Even after 4 days not more than 50 per cent of either dextrin had been completely hydrolyzed. Also, the hydrolyzed fragments with activating ability appeared to be fairly stable in sulfuric acid under these conditions.

Likewise, the hydrolysis of amylose by 11 N sulfuric acid was much slower

than when 7.5 N hydrochloric acid was used. Lower concentrations of the former acid were even less effective. When 11 N sulfuric acid was used at 24°, approximately 40 hours were required to attain the maximum activating effect. In addition, the rate of decline in activating ability was very gradual. 10 days after the maximum had been reached the activating power had declined approximately 40 per cent. 7 days later it had not undergone further decline. The behavior of amylose and of Schardinger dextrins in sulfuric acid may be of considerable practical value in isolating short chain dextrins with high activating ability.

SUMMARY

The ability of different polysaccharides, dextrins, and certain di- and trisaccharides to activate potato phosphorylase was determined. Amylopectin from corn-starch had the greatest activity. Melezitose and raffinose, which are trisaccharides, had no activity.

Hydrolysis of amylose and of amylopectin by β-amylase caused a progressive decrease in activating power. On the other hand, hydrolysis of amylose with either HCl or H₂SO₄ markedly increased its activity.

The effect of α- and β-Schardinger dextrins in inhibiting the activating ability of polysaccharides was confirmed. Partial hydrolysis of these dextrins with either HCl or H₂SO₄ caused them to be activators of potato phosphorylase.

Certain phosphorylase activators are fairly stable in strong H₂SO₄.

It is concluded that activators of potato phosphorylase need not contain more than 6 or 7 glucose units per molecule and that the Schardinger dextrins are promising sources of material for the continuation of investigations on the nature of the activators of potato phosphorylase.

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VITAMIN CONTENT OF MOUSE EPIDERMIS DURING METHYLCHOLANTHRENE CARCINOGENESIS

I. BIOTIN, CHOLINE, INOSITOL, p-AMINOBENZOIC ACID, AND PYRIDOXINE*

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In the Barnard Free Skin and Cancer Hospital project to analyze the carcinogenic effect of methylcholanthrene on mouse epidermis (1), data on the levels of certain inorganic constituents and of ascorbic acid (2, 3) and of lipids (4, 5) in the reacting epidermis have been published. Although a number of investigators have found differences between normal and cancerous tissues in certain vitamins of the B group (6–9), these important constituents have not previously been determined either in isolated epidermis or during controlled methylcholanthrene carcinogenesis. Accordingly, vitamin bioassay methods, with mutant strains of *Neurospora* (10), have been developed or modified for use in measuring the epidermal content of certain vitamins during methylcholanthrene carcinogenesis. The results of a considerable number of determinations suggest that the application of methylcholanthrene in benzene solution does not significantly alter the levels of inositol, choline, or p-aminobenzoic acid in mouse epidermis. A slight increase in pyridoxine content seems to follow treatment with methylcholanthrene (to 124 per cent of normal) and also with benzene alone (to 118 per cent of normal). The most striking specific effect of methylcholanthrene is an apparent decrease in the biotin content to 64 per cent of normal.

EXPERIMENTAL

Material—Female Swiss mice, 3 to 6 months old, were used, except as indicated in Table III. The animals, in groups of thirty, were shaved over the entire back, only distilled water being used to wet the hair. After a 2 or 3 day interval, they were painted over the shaved areas either with reagent grade benzene or with a 0.6 per cent solution of 20-methylcholanthrene in benzene, a No. 5 camel's hair brush being used, as previously de-

scribed (4). The benzene-treated controls and the methylcholanthrene-treated mice were painted three times a week, different groups receiving one, three, six, thirteen, or twenty-five paintings. The groups painted one or three times were killed with chloroform 10 days after the first painting; those painted six, thirteen, or twenty-five times were killed respectively 20, 30, and 60 days after the first painting, while the untreated controls were killed 10 days after they were shaved. Before any of the animals were killed, they were dry-shaved, if necessary, to remove any regrown hair. In all cases the epidermis was separated by blunt dissection at 50°, according to the method of Baumberger, Suntzeff, and Cowdry (11). The pooled sample of epidermis from each group of thirty mice was dried *in vacuo* over phosphorus pentoxide at room temperature in the dark for a minimum of 60 hours, and was stored under the same conditions. The dry weights of the pooled samples ranged from 0.4 to 0.8 gm.

Extraction—Inositol and choline and most of the rest of the B vitamins are present in tissues in the bound form and must be in solution for assays. Liberation with acid was selected in preference to enzyme treatment (12), since acid hydrolysis is necessary for choline (13), and should be equally effective for biotin (14), p-aminobenzoic acid (15), and inositol (16). For liberation of pyridoxine, 1 N hydrochloric acid for 1 hour (17) and 2 N sulfuric acid for ½ hour (18) have been recommended. However, pyridoxine has been shown to stand autoclaving with 4 N sulfuric acid for ½ hour (19). Preliminary assays of epidermis gave as high or higher values for all five vitamins after treatment with 4 N sulfuric acid and neutralization with sodium bicarbonate as after 1 N sulfuric acid and neutralization with barium hydroxide (13). Under the conditions used, the salt concentration was found not seriously to interfere with the assays. Treatment with 4 N sulfuric acid for 2 hours at 15 pounds pressure was therefore selected for liberation of all five substances. After autoclaving with 1 cc. of acid per 0.1 gm. of dry epidermis, each extract was diluted with water, neutralized to Congo red with sodium bicarbonate, filtered, made up to standard volume (1 cc. per 6 mg. of dry epidermis), and adjusted to pH 5.5. The solutions were placed in flasks, a few drops of toluene added, and the flasks corked and stored at 10° until needed. Assays run on samples stored up to 1 month showed no significant alterations in the values for these five substances.

General Assay Procedure—All glassware used for assays or for storage of samples was routinely cleaned with chromic-sulfuric acid mixture and thoroughly rinsed. Different mutant strains of *Neurospora* were used for the assays. These strains had been produced by x-ray and ultraviolet light treatment and each requires for growth a specific vitamin (10, 20). They can be used for bioassays of these vitamins by measuring the growth in

liquid medium (13).   The growth of each strain is a function of the concentration of the required vitamin added.   The minimal medium previously described (13) was made up in twice the final desired concentration, sterilized, and diluted as required.   10 cc. lots of medium containing the desired supplements were placed in 50 cc. Erlenmeyer flasks, as used by Stokes *et al.* (17), plugged with purified absorbent cotton, and sterilized for 10 minutes at 15 pounds pressure.   Each flask of medium was then inoculated with 1 drop of a water suspension of conidia of the appropriate strain of *Neurospora*.   Cultures grown on agar slants and not over 14 days old were used to prepare the inoculum.   The flasks were incubated at 25° for 72 hours without shaking.   At the end of this period, the mycelium was removed from the flask, placed on filter paper in a Büchner funnel, washed well with distilled water, removed from the filter paper, and dried in an oven at 90°

TABLE I

Range of Vitamin Concentrations Effective for Strains of Neurospora

Vitamin	Strain	Effective vitamin range per flask (10 cc. of medium)	Bibliographic reference to other *Neurospora* assays
		γ	
Biotin....................	*N. crassa* 1	0.00005– 0.001*	(21)
Choline...................	" " 34486	0.5 – 5.0	(13, 21–23)
Inositol..................	" " 37401	2.0 –10.0	(24)
p-Aminobenzoic acid........	" " 1633	0.01 – 0.05	(15, 25)
Pyridoxine................	" *sitophila* 299	0.005 – 0.07†	(17)

* Biotin-free minimal medium.
† Minimal medium containing 3 γ of thiamine per 10 cc.

for 2 hours.   The dried mycelium was then weighed to the nearest 0.1 mg. directly on the pan of an analytical balance.

Specific Assay Procedure—The strain of *Neurospora* used for each assay and the effective range of vitamin concentrations under the adopted conditions are given in Table I.   Inositol was determined with *N. crassa* 37401 essentially as described by Beadle (24).   Strain 1633 (15, 25) was used for the assay of p-aminobenzoic acid.   The wild type strain of *N. crassa* (strain 1) was used for the determination of biotin by the same procedure as the other assays except that biotin-free medium was used.   This was prepared in the same manner as the other minimal medium but with omission of biotin[1] and with the use of sucrose which had been treated in 20 per cent solution with one-tenth of its weight of norit.   (Hodson (21) has found the *cholineless Neurospora crassa* (strain 34486) satisfactory for biotin assays as well as for choline assays.)

[1] The biotin used in this work was kindly supplied by Merck and Company, Inc., Rahway, New Jersey.

Pyridoxine was determined with *Neurospora sitophila* strain 299 essentially as reported by Stokes *et al.* (17), but instead of destroying thiamine in the extracts tested, an excess of thiamine (3 γ per 10 cc.) was added to the basal medium. Under these conditions, the stimulating effect of thiamine on the response of strain 299 to pyridoxine (26) increases the sensitivity of the assay, and incubation at 25° for 3 days is then adequate. The growth response to limiting amounts of pyridoxine was observed to be maximal and constant in the presence of from 1 to 6 γ of thiamine per 10 cc. of medium.

TABLE II

Within Series Reproducibility of Vitamin Assays and Recoveries of Added Vitamins

Vitamin	Epidermal extract	Vitamin added	Duplicate dry weight of mold	Vitamin found		Recovery of added vitamin	
				Per flask	Per cc. extract		
	cc.	γ	*mg.*	γ	γ	γ	*per cent*
Biotin	0.2	0	7.7, 8.2	0.000195	0.00098		
	0.4	0	12.2, 11.8	0.00038	0.00095		
	0.6	0	15.4, 16.4	0.00060	0.0010		
	0.2	0.0001	10.4, 9.6	0.00030		0.000105	105
Choline	0.1	0	7.0, 7.2	1.5	15.0		
	0.3	0	16.2, 16.3	4.55	15.2		
	0.5	0	20.4, 22.2	7.2	14.4		
	0.1	1.0	10.3, 10.5	2.5		1.0	100
Inositol	0.5	0	1.9, 2.0	1.9	3.8		
	1.0	0	4.7, 4.7	4.15	4.15		
	2.0	0	9.0, 9.2	6.75	3.4		
	0.5	3.0	6.1, 6.1	5.0		3.1	103
p-Aminobenzoic acid	1.0·	0	4.9, 4.6	0.019	0.019		
	2.0·	0	9.7, 9.4	0.028	0.014		
	1.0	0.02	14.7, 15.8	0.038		0.019	95
Pyridoxine	0.5	0	4.8, 5.4	0.0085	0.017		
	1.0	0	9.0, 8.6	0.019	0.019		
	0.5	0.01	8.3, 9.6	0.0195		0.011	110

Neurospora crassa strain 34486 was used for the determination of choline essentially as described by Horowitz and Beadle (13), except that permutit adsorption of the extract was omitted. Preliminary assays of epidermis gave the same choline values with this procedure as with the hydrolysis and adsorption procedure of Horowitz and Beadle (see also Hodson (21), Luecke and Pearson (22), and Siegel (23)). The choline content of epidermis is apparently so high in relation to that of methionine that the removal of the latter is unnecessary.

A standard curve was made for every run of each vitamin, with all con-

centrations in duplicate. For each sample of epidermis assayed, two or three levels of extract, in duplicate, were used for each determination. The amount of the vitamin in each extract was then estimated from the standard curve. Typical results are given in Table II. In all assays the vitamin contents per gm. of dry epidermis calculated from the growth at different levels of extract were in good agreement, and the recoveries of added vitamins were satisfactory. The reliability of the extraction and assay techniques was tested by repeating the assays on several extracts at different times up to several weeks apart, and by assaying independently prepared extracts of the same samples of epidermis. In both cases the results agreed within the limits allowable for a bioassay.

<div align="center">TABLE III</div>

Vitamin Content of Mouse Epidermis during Methylcholanthrene Carcinogenesis
The values given are in micrograms per gm. of dry epidermis, for the number of groups indicated in parentheses.

Treatment	No. of paintings	Biotin	Choline	Inositol	p-Amino-benzoic acid	Pyridoxine
		γ	γ	γ	γ	γ
Normal*		0.196 (5)	2471 (5)	526 (5)	2.40 (5)	2.45 (5)
Benzene	1	0.197 (2)	2625 (2)	539 (2)	2.19 (2)	2.70 (2)
	3	0.200 (2)	2630 (2)	536 (2)	2.55 (2)	2.57 (2)
	6	0.232 (2)	3315 (2)	632 (2)	2.10 (2)	3.49 (2)
	13	0.163 (3)	2636 (2)	568 (2)	2.33 (3)	2.76 (2)
Methylcholanthrene	1	0.116 (2)	2640 (2)	535 (2)	2.32 (2)	2.72 (2)
	3	0.122 (3)	2270 (3)	516 (3)	2.02 (3)	2.96 (3)
	6	0.132 (2)	2925 (2)	569 (2)	2.92 (2)	2.95 (2)
	13	0.124 (2)	2820 (2)	547 (2)	2.67 (2)	3.29 (2)
	25	0.136 (2)	2791 (2)	582 (2)	2.14 (1)	3.45 (2)

* Includes two lots of mixed Swiss and New Buffalo mice.

Results

Tables III and IV present the results obtained with groups of mice painted with benzene up to thirteen times over a period of 1 month and with groups painted with a 0.6 per cent solution of 20-methylcholanthrene in benzene up to twenty-five times over a period of 2 months. Dry weight of epidermis, rather than wet weight, was used as the basis of reference, since in separation of the epidermis from the dermis at 50° there was a variable loss of water from the tissue.

There was no change in the values for p-aminobenzoic acid following the application of either benzene alone or methylcholanthrene in benzene. Choline and inositol values showed a slight increase in the methylcholan-

threne-treated mice with a somewhat greater increase in the benzene control mice.   In the benzene controls, the choline averaged 113 per cent of the normal and the inositol 108 per cent of normal.   However, as is shown in Table II, the method of assay has an error of at least 10 per cent and therefore no conclusions can be drawn from these small changes in value.   The pyridoxine values showed a somewhat greater increase, to 118 per cent of normal in benzene-treated mice and to 124 per cent in methylcholanthrene-treated mice.

Of the vitamins studied, the biotin content showed the most marked change, the average for the mice treated with methylcholanthrene in benzene being 64 per cent of normal, while the average for the benzene controls was 99 per cent of normal.   With methylcholanthrene painting the biotin content was slightly lower after one painting than it was after a number of paintings.   Although the average biotin value for all of the benzene controls was 99 per cent of normal, benzene alone may have some depressing

TABLE IV

Summary of Vitamin Content of Mouse Epidermis during Methylcholanthrene Carcinogenesis

The values are averages in per cent of the values for normal epidermis, for the number of groups indicated in parentheses.

Treatment of epidermis	Biotin	Choline	Inositol	p-Amino-benzoic acid	Pyridoxine
Benzene................	99 (9)	113 (8)	108 (8)	96 (9)	118 (8)
Methylcholanthrene..........	64 (11)	107 (11)	104 (11)	100 (10)	124 (11)

effect upon the biotin content of the epidermis, since after thirteen benzene paintings the biotin value was 83 per cent of normal.

DISCUSSION

The validity of the observed variation in the biotin content of mouse epidermis during methylcholanthrene carcinogenesis is perhaps supported by the consistency of the results obtained for the other vitamins determined and for biotin in normal epidermis.   The fall in biotin occurs rapidly, after only a single application of methylcholanthrene, and is in this respect analogous to the behavior of the iron and calcium (2) and the total fat (4).   This lowered biotin content may result from altered biotin metabolism.   Burk and Winzler have suggested that the metabolism of biotin vitamers may be abnormal in tumors (27).

Although the slight increases in pyridoxine content with methylcholanthrene treatment are not statistically significant, further determinations

may support the apparent trend toward higher pyridoxine levels and make possible an appraisal of the validity of the rise with benzene treatment alone. The pyridoxine values represent "total B_6" content, since *Neurospora* responds equally well to pyridoxine, pyridoxal, and pyridoxamine (28). Assays for specific vitamin B_6 derivatives in methylcholanthrene-treated epidermis might be quite illuminating in regard to a possible change in pyridoxine metabolism.

Changes in both biotin and pyridoxine levels might be associated with changes in nitrogen metabolism, since both biotin (29) and pyridoxine (30–32) seem to be concerned with nitrogen metabolism.

Finally, it should be emphasized that the biochemical changes reported in this and previous communications from this laboratory (2–5) represent only steps toward the development of a complete, unified picture of all the biochemical changes taking place in epidermis during methylcholanthrene carcinogenesis, and that only the complete picture may enable the relation of any changes to the development of malignancy.

SUMMARY

By the use of mutant strains of *Neurospora* the levels of biotin, choline, inositol, *p*-aminobenzoic acid, and pyridoxine have been followed during methylcholanthrene carcinogenesis.

No significant changes in inositol, choline, or *p*-aminobenzoic acid were observed. The pyridoxine content appeared to increase slightly after either methylcholanthrene or benzene treatments.

The biotin content was found to decrease after methylcholanthrene treatment, reaching a level of 64 per cent of normal after a single application of the carcinogen, and maintaining approximately the same level during repeated paintings over a period of 60 days.

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THE HYDROLYSIS OF HYDANTOIN BY VARIOUS TISSUES

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In 1912 Lewis (1) administered hydantoin to rabbits, cats, and dogs and came to the conclusion that the ring was not readily broken in the body. Gaebler and Keltch (2) some years later showed that some hydantoin was excreted as hydantoic acid after injection into dogs. Wada (3) claimed that urea was produced from hydantoin after incubation with milk or certain tissue suspensions. Finally, Kozelka and Hine (4) gave diphenyl-hydantoin to men and dogs and found that 1 to 5 per cent was excreted as the corresponding hydantoic acid and 10 to 27 per cent as α-aminodiphenyl-acetic acid. About 30 per cent of the drug was unaccounted for. It was therefore of interest to investigate the metabolism of hydantoin and the diphenyl derivative in detail by *in vitro* methods.

EXPERIMENTAL

The first experiments were done with rat liver slices suspended in Krebs' bicarbonate solution. 300 mg. of slices (wet weight) were suspended in 4.0 cc. of solution and placed in 50 cc. Erlenmeyer flasks in an atmosphere of 95 per cent O_2 and 5 per cent CO_2 or 95 per cent N_2 and 5 per cent CO_2. 2.0 to 4.0 mg. of hydantoin or diphenylhydantoin (kindly supplied by Dr. E. A. Sharp of Parke, Davis and Company) were added and the vessels incubated for 3 to 4 hours at 37°. At the end of this time 1.0 cc. of 20 per cent trichloroacetic acid was added to each vessel and the precipitated protein was centrifuged down. An aliquot of the supernatant fluid was subjected to Archibald's (5) procedure for citrulline, which gives no color with the hydantoins but does with the hydantoic acids, and another aliquot to Bonsnes and Taussky's (6) creatinine method which gives a color with hydantoins but not with the coresponding acids. By these methods the following facts were established. Hydantoin is rapidly hydrolyzed by rat liver slices, and less rapidly by kidney. The hydrolysis occurs equally well with or without oxygen, and in the presence of cyanide. Diphenyl-hydantoin, however, is not hydrolyzed. It is very insoluble and possibly the negative result is due to an inadequate concentration of substrate in solution. To certain of the vessels in which hydantoin had been incubated a solution of urease was added at the end of the experiment before the addition of the trichloroacetic acid. After 30 minutes incubation at 40°

the solutions were tested both for the presence of hydantoic acid and ammonia, the latter by a vacuum distillation method. · The results showed that no urea was formed either aerobically or anaerobically.

When these facts had been established, tissue supensions were substituted for slices. Rat liver suspensions hydrolyze hydantoin rapidly and the hydantoic acid produced will displace carbon·dioxide from solution, thus making it possible to obtain accurate measurements of the rate of reaction. The suspensions were made in 0.05 M phosphate buffer of pH 7.8 containing
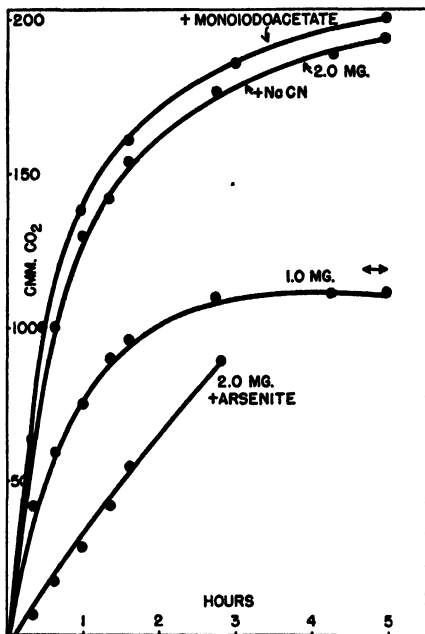
FIG. 1. The hydrolysis of 1.0 and 2.0 mg. of hydantoin by dialyzed rat liver suspension, pH 7.8, 37°, and the effect of monoiodoacetate, cyanide, and arsenite on the rate of hydrolysis of 2.0 mg. The double arrow represents the theoretical CO_2 production for the hydrolysis of 1.0 mg. of hydantoin to hydantoic acid.

0.05 M sodium bicarbonate and were equilibrated in Warburg vessels with 95 per cent N_2 and 5 per cent CO_2. The hydantoin was added from the side arm and the CO_2 evolution measured in the usual way. Fig. 1 shows that the theoretical amount of CO_2 is displaced and that the amount is proportional to the concentration of hydantoin.

The distribution of the enzyme in various organs and animals was first studied. It is active in rat liver and less active in rat kidney, but is absent from blood and brain. It is present in dog liver and kidney, cat kidney, questionably in cat liver, and in frog liver. It is absent from dog and cat

blood, mouse liver, rabbit and guinea pig liver, kidney, and blood, and frog kidney. 100 mg. of pancreatin (Merck) do not hydrolyze hydantoin. With the exception of the mouse, it seems that the enzyme is present in omnivores and absent in herbivores. Neither diphenylhydantoin nor barbituric acid is hydrolyzed by any of these tissues.

The characteristics of the enzyme in rat liver were studied. The tissue suspension can be dialyzed for 18 hours without loss of activity. Precipitation of the suspension with half saturation of ammonium sulfate or 50 per cent alcohol and subsequent dialysis yields only inactive fractions. Precipitation with acetone causes approximately a 50 per cent loss in activity. The rate of hydrolysis in dialyzed liver suspension is not affected by 0.4×10^{-2} M sodium cyanide, 0.6×10^{-2} M cysteine, 0.3×10^{-2} M monoiodoacetate, 3.6×10^{-2} M sodium fluoride, nor a saturated solution of diphenylhydantoin, but is about 80 per cent inhibited by 0.2×10^{-2} M sodium arsenite. These results are shown in Fig. 1. The enzyme has no sharp pH maximum in the range studied. Thus the rates of hydrolysis at pH 6.7, 7.8, and 8.6 are within 10 per cent of each other.

DISCUSSION

As far as we are aware the known peptidases do not hydrolyze hydantoins and this suggests that a specific enzyme is involved. Its function in the animal is not clear, for hydantoins do not occur in nature. It is possible that substituted hydantoins are also hydrolyzed by the enzyme, as suggested by the results of Kozelka and Hine with diphenylhydantoin. The failure to show hydrolysis of this latter compound *in vitro* may simply be due to its insolubility in water at physiological hydrogen ion concentrations.

SUMMARY

The distribution and properties of an enzyme that hydrolyzes hydantoin have been described.

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TRANSAMINATION IN GREEN PLANTS

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The transamination reaction discovered by Braunstein and Kritzmann (1) in 1937 seemed, according to their investigations performed with animal tissues, to explain the formation of all amino acids in them. In this laboratory, the reaction has been under investigation during the elucidation of the synthesis of amino acids in green plants, in which the said reaction was demonstrated by Virtanen and Laine (2) in 1938. It was noted in the system $l(+)$-glutamic acid + oxalacetic acid $\rightleftarrows$ α-ketoglutaric acid + $l(-)$-aspartic acid, and also in the systems l-aminodicarboxylic acids + pyruvic acid and l-aminodicarboxylic acids + α-ketoisocaproic acid. In the former case the reaction was strong; in the latter weak. Hydroxyglutamic acid did not transfer its amino group with pyruvic acid. When phenylpyruvic acid was the other component with l-aminodicarboxylic acids, no transfer of amino groups occurred (3).

I have examined the transamination in green plants further, both with crushed plant material and by the vacuum infiltration method, and have noted that many different plant tissues contain agents which catalyze the reaction.

Analytical Methods

Aminodicarboxylic acids were precipitated according to the method of Foreman. Aspartic acid was determined, after precipitation according to Foreman, with the malic acid method of Pucher (4) adapted by Arhimo (5) to aspartic acid.

Results

In systems in which l-glutamic acid or α-ketoglutaric acid acted as the dicarboxylic acid component, the reaction maximum was reached in 40 to 60 minutes. When l-aspartic acid or oxalacetic acid was the dicarboxylic acid component, the reaction was slower and the percentage of transamination lower (Fig. 1). In systems of both kinds, the optimum pH was about 6.9 (Fig. 2) and the optimum temperature 41° (Fig. 3).

In the following combinations, the rate of reaction was found to decrease in the following order.

(1) α-Ketoglutaric acid + $l(-)$-aspartic acid $\rightleftarrows$

$l(+)$-glutamic acid + oxalacetic acid

(2) α-Ketoglutaric acid + $l(+)$-alanine $\rightleftarrows$ $l(+)$-glutamic acid + pyruvic acid

(3) Oxalacetic acid + $l(+)$-alanine $\rightleftarrows$ $l(-)$-aspartic acid + pyruvic acid

(4) α-Ketoglutaric acid + $l(+)$-valine $\rightarrow$ $l(+)$-glutamic acid + α-ketovaleric acid

The reaction last mentioned was weak.

With the following acid combinations transamination could not be demonstrated: $l(+)$-glutamic acid + phenylpyruvic acid, α-ketoglutaric acid + $l(-)$-tyrosine, $l(-)$-aspartic acid + phenylpyruvic acid, oxalacetic acid + $l(-)$-tyrosine, oxalacetic acid + $l(+)$-valine.

Virtanen and Laine stated that the important amides in plants, glutamine and asparagine, are not able to transfer the amino group.
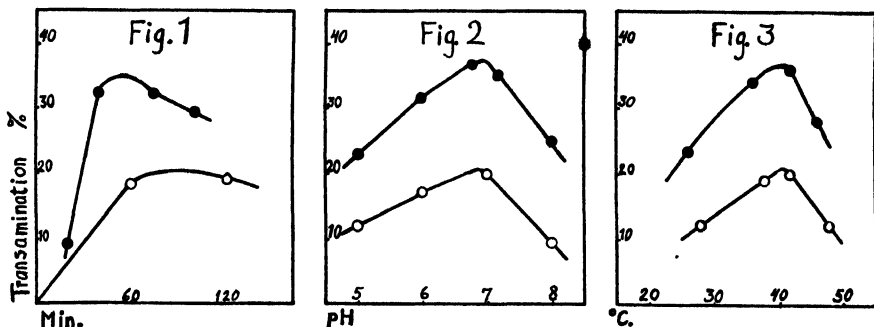
FIGS. 1 TO 3. The solid circles represent glutamic acid + pyruvic acid; the clear circles, aspartic acid + pyruvic acid.

It is apparent that the formation of certain aliphatic amino acids, notably of alanine, occurs through transamination in plants. On the other hand, in the formation of aromatic amino acids, this reaction mechanism does not seem to have any direct influence. Accordingly, these amino acids must be formed through some other mechanism. It is interesting to emphasize in this connection that most amino acids can be regarded as derivatives of alanine.

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PRESSURE AND PROTEIN DENATURATION

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Kinetic analyses have indicated that moderate hydrostatic pressures, up to some 700 atmospheres, oppose reversible and irreversible denaturations of certain enzyme systems, apparent at temperatures above the normal optimum of the enzyme reaction, as well as at lower temperatures in the presence of denaturants such as alcohol (1–4). Qualitative observations have shown that such pressures also retard the precipitation of highly purified human serum globulin and egg albumin at 65° (5) and slow the destruction of specific antitoxic activity at the same temperature (6). In this study we have obtained quantitative data with regard to the influence of various pressures, up to 10,000 pounds per sq. in., and of low concentrations of ethyl alcohol on the time course of precipitation of human serum globulin[1] at 65° and pH 6.0.

EXPERIMENTAL

Methods

Solutions of approximately 2 per cent globulin were made in 0.8 per cent NaCl containing 0.01 M phosphate buffer, pH 6.0. Portions of the stock solution were generally diluted to one-third with the buffered salt solution, and then distributed in 100 × 13 mm. test-tubes. For denaturation at normal pressure, the tubes were stoppered and placed in a water bath at 65° ± 0.02°. Similar tubes were completely filled with portions of the same solution, closed with rubber stoppers, and placed in a water-filled, steel pressure chamber which was then attached to a hydraulic pump. The desired pressure was applied and the entire chamber was placed in the same water bath. Temperature equilibration required 2½ minutes outside, and 5½ minutes inside the pressure chamber. The lag

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in heating, for which allowance in timing was always made, introduced a source of error because of the changing rates of reaction with rise in temperature. Because of the high temperature coefficient of denaturation, however, this error was small, except with very brief periods of heating. Substantial agreement (within 5 per cent) was obtained between the amount of precipitate in tubes heated for an equivalent period of 30 minutes outside the pressure chamber and inside the chamber under a pressure of only 100 pounds per sq. in. At the end of the designated period of heating, the pressure chamber was placed in a water bath at room temperature, which very quickly cooled the specimens to a temperature which gave rise to no appreciable further denaturation. The tubes that were not subjected to pressure cooled sufficiently rapidly in air on removal from the 65° water bath.

After being heated, the solutions were centrifuged for 20 minutes at 25,000 times gravity, while the rotor was maintained at a low temperature by dry ice placed on the top of the centrifuge. The precipitates were discarded and the supernatants analyzed for the nitrogen in solution by micro-Kjeldahl determinations. In most cases the supernatant showed a distinct opalescence. This was much less noticeable in the specimens containing alcohol. The error caused by the presence of small amounts of denatured protein remaining in suspension was very small, however, except when the total amount of denaturation was slight, as with short periods of heating such as 5 to 10 minutes, or with longer periods of heating under high pressures.

Influence of Initial Protein Concentration and Products of Denaturation on Time Course of Reaction

With solutions containing between 2.0 and 0.2 per cent protein at the start, the percentage of the original quantity of protein remaining in solution after 30 minutes at 65° did not vary significantly with the initial concentration. The amount of protein precipitated in this time was usually about 50 per cent of that initially present, with some variation, for reasons not entirely clear, in repeated experiments with different solutions made up from the same lot of the dry globulin. Fig. 1 represents the course of protein denaturation in three solutions prepared as follows: Solution A, from which the other two were prepared, contained 1.85 per cent protein. A portion of Solution A was diluted to a concentration of 0.7 per cent protein, giving Solution B. Solution C was obtained by heating a portion of Solution A for 30 minutes at 65°, centrifuging, discarding the precipitate, and saving the supernatant. The three solutions were stored for 1 week at 3–6°, and then treated at 65° simultaneously. Fig. 1 shows that both the undiluted (Solution A) and diluted (Solution B)

solutions follow the same curve for the percentage of the initial quantity of protein precipitated with time. The partially denatured Solution C, from which the precipitate had been removed, indicates very much the same curve, when the period of preheating and amount of precipitate that was removed earlier are taken into account. The same data for this solution, computed on the basis of the amount of protein in solution at the start of the second heating as 100 per cent, give the uppermost curve, which
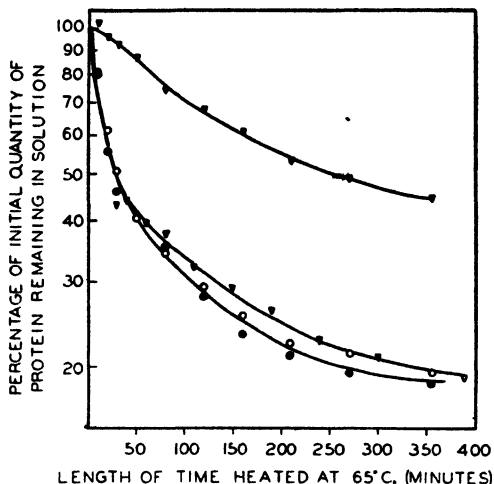
FIG. 1. Denaturation of human serum globulin at 65°. Solid circles, Solution A (see the text) containing globulin in a concentration of 1.85 per cent, before treatment; open circles, Solution B, obtained by diluting Solution A to an initial concentration of 0.7 per cent protein in solution. The triangles refer to Solution C, the supernatant of a portion of Solution A that had been partially denatured by being heated for 30 minutes at 65°. It was then centrifuged, the precipitate was discarded, and the supernatant was stored for 1 week at 3–5°. The solid triangles show the course of precipitation in Solution C with the protein in solution at the start of the second heating taken as 100 per cent. The open triangles illustrate the same data, but take into account the time of denaturation and amount of protein precipitated in the first heating, with the original concentration of 1.85 per cent protein as 100 per cent. The scale of the ordinate is logarithmic.

shows a slight lag and the lack of a relatively rapid denaturation during the first 30 minutes. In other experiments, in which the solutions were first heated for 30 minutes at 65°, then maintained at room temperature for periods of 12 minutes to 4 hours without removal of the precipitate and again heated at 65°, the same curve as that observed for continuously heated solutions was obtained.

The foregoing results indicate that the specific rate of denaturation is first order with respect to the initial amount of protein, and is essentially

independent of the products of the reaction. The shape of the curve, however, shows that the reaction is not unimolecular, but is more complex, as is evidenced by the decreasing rate with time. In these respects the over-all reaction resembles that for the denaturation of diphtheria antitoxin by urea (7) at room temperature, and for the denaturation of anti-*Staphylococcus* hemolysin at 65° under both normal and increased pressure (6). The general shape of the curve is also similar to that for the denaturation of tetanus antitoxin at 65° (8).
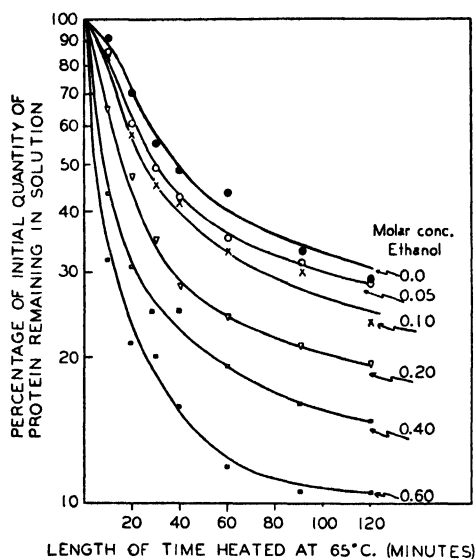
FIG. 2. Influence of alcohol on the rate of precipitation at 65° of globulin solutions having a concentration of 0.69 per cent protein before treatment at 65°. These concentrations of alcohol caused no visible precipitation over long periods of time at room temperature. Logarithmic scale on the ordinate.

Influence of Alcohol—Small concentrations of ethyl alcohol increase the rate of precipitation, as is illustrated by the data in Fig. 2 from an experiment with a single stock solution of globulin. The concentration of alcohol required to bring about a precipitation of the protein at room temperature is of the order of 10 times the concentrations that markedly increase denaturation at 65°. The shape of the curve for each concentration is the same, however, if the time scale is changed, as is shown by the straight lines which result when the logarithm of the proportion of the protein originally in solution is plotted against the logarithm of the time of denaturation. These lines have practically the same slope but differ in position on the abscissa (Fig. 3). Thus, in accord with the ob-

servations of Lepeschkin (9) on the similarity of the heat and alcohol denaturation of egg albumin, as well as the recently studied effects of alcohol on the luminescent system (4), the action of alcohol appears to be essentially that of accelerating the denaturation reaction. Preliminary analyses, based on the relation between the logarithm of the concentration of alcohol and the logarithm of the amount of acceleration, indicate that an average of approximately 1.5 more molecules of alcohol are combined with the activated protein molecule undergoing denaturation than with the
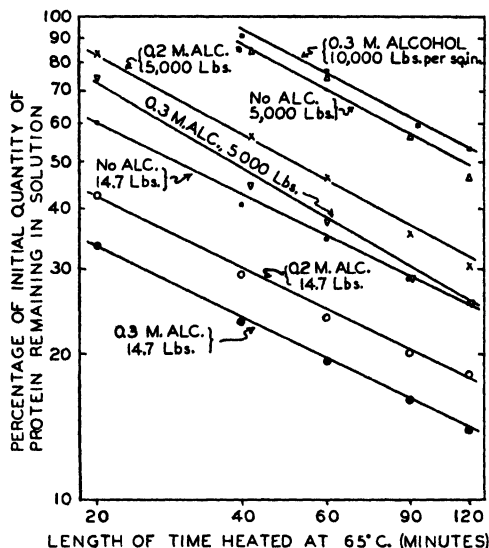
FIG. 3. The data of Figs. 4 to 7, plotted on logarithmic scales as the percentage of the quantity of protein in solution before being heated, against the time of heating at 65°, with and without alcohol, under normal and increased hydrostatic pressure, respectively. Only representative curves are shown to avoid crowding. The scale is logarithmic on both the ordinate and abscissa.

normal molecule. A definite value for this ratio must await clarification of the reason for the apparent change in rate of precipitation during the course of the reaction.

Action of Hydrostatic Pressure—Hydrostatic pressure retards the rate of precipitation both in the presence and in the absence of alcohol. The results of a series of experiments with pressures up to 7500 pounds per sq. in. are shown in Figs. 4 to 6, in which the curve for normal pressure in each case represents the average of five repeated determinations. Fig. 7 shows the results of a single experiment with reference to the effects of 10,000 pounds pressure in comparison with atmospheric, and with alcohol concentrations of 0, 0.2, and 0.3 M, respectively. In all cases, the effect of

alcohol is to increase, while that of pressure is to decrease the rate of precipitation. High pressures cause a conspicuous lag in precipitation, which hardly represents a complete initial prevention of denaturation, but rather a very pronounced slowing of a reaction, whereby the concentration of denatured molecules in solution builds up enough to form a precipitate. The source of error referred to earlier no doubt tends to exaggerate this effect; *i.e.*, with very small amounts of precipitate, it is difficult to clarify the solution in the centrifuge. Moreover, a slight evaporation of the solu-

FIG. 4.

FIG. 5.
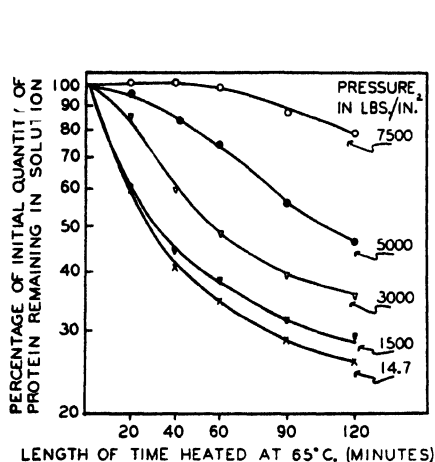
FIG. 4. The influence of hydrostatic pressure on the rate of precipitation without added alcohol. The curve at normal pressure is the average of five repeated experiments. The data for each of the curves are from a separate pressure experiment. Initial concentration of globulin in the different experiments, from 0.74 to 0.85 per cent. Logarithmic scale on the ordinate.
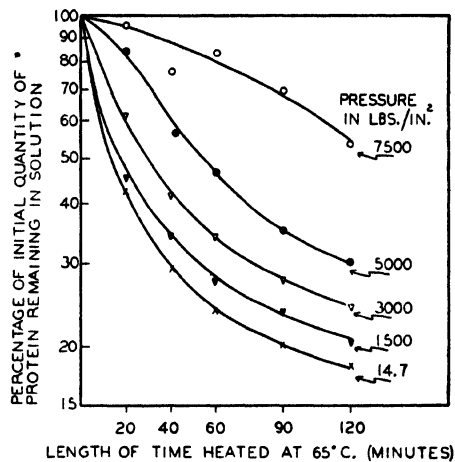
FIG. 5. The effects of hydrostatic pressure on the rate of precipitation at 65° of globulin solutions containing 0.2 M ethyl alcohol. The curve for normal pressure represents the average of five repeated experiments, the others a single experiment. Logarithmic scale on the ordinate.

tion during the centrifugation would contribute in the same direction to this error. At lower pressures, the rapidity of denaturation makes it difficult to obtain accurate observations during the first few minutes, but when data including the amount of denaturation at the end of the first 10 minutes are plotted with somewhat broader spaces per time unit on the abscissa (*e.g.*, Fig. 2), it is apparent that there is a period of slow precipitation at the start, followed by more rapid precipitation, and a subsequent slowing (*cf.* also Fig. 3).

The changing rates of precipitation with time of heating may be accounted for either by a heterogeneity of the protein molecules, the net
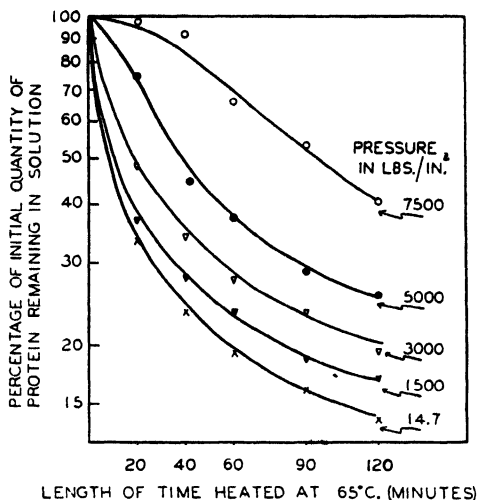
FIG. 6. Influence of hydrostatic pressure on the rate of precipitation at 65° of globulin solutions containing 0.3 M ethyl alcohol. The curve at normal pressure represents the average of five experiments, the others a single experiment. Logarithmic scale on the ordinate.
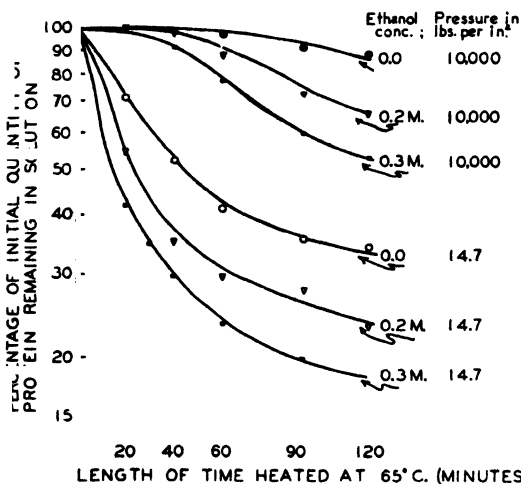
FIG. 7. The rate of precipitation at 65° of globulin solutions, in a concentration of 0.69 per cent protein at the start, containing 0, 0.2, and 0.3 M alcohol, respectively, under normal and 10,000 pounds per sq. in. hydrostatic pressure. The data are from a single experiment. Logarithmic scale on the ordinate.

result representing the summation of reactions with different specific rate constants, or as the result of a complex of reactions with homogeneous molecules, or both. The effects of high pressure in greatly prolonging the

initial time required for the formation of an appreciable amount of precipitate suggest that the first step involves a denaturation reaction with a very large volume change of activation (10).   The following experiment provides evidence that a subsequent reaction, possibly an equilibrium, which is characterized by a small volume increase of the final over the initial states, is involved in the precipitation of the denatured protein.

Tubes containing an initial concentration of 0.686 per cent globulin with 0, 0.2, and 0.3 M alcohol, respectively, were treated in triplicate at 65° for 40 minutes.   One set was cooled to room temperature, a second set to 4°, in both cases under atmospheric pressure, and the third set to 4° under 10,000 pounds pressure.   After 21 hours, the tubes at room temperature were analyzed in the usual manner.   The specimens which had been kept at 4° were centrifuged in a rotor precooled approximately to this temperature, and maintained at a low temperature throughout the centrifugation. The results are summarized in Table I, which indicates first that, as might be expected, there is an increase in flocculated protein at low temperatures, and second, that a high pressure opposes this increase.   Moreover, the amount of precipitate that has already formed at room temperature may be decreased by subjecting the specimens to high pressure over a long period of time, as is shown by the data in Table II, which are taken from an experiment with a similar series of triplicate tubes.   Denaturation was first carried out at atmospheric pressure for 20 minutes at 65° and all tubes were cooled to room temperature.   The first set was centrifuged and analyzed very shortly thereafter.   The other two sets were maintained for 64 hours at room temperature, one set under normal pressure, the other under 10,000 pounds.   From Table II, it is apparent that, within the limits of experimental error, the amount of precipitate did not increase on long standing at room temperature.   On the other hand, a significant decrease in the amount of precipitate occurred as a result of high pressure. It is possible, of course, that this effect takes place to some extent through a reversal of the denaturation reaction, but it would seem more likely that it represents an action of pressure on the flocculation of the denatured molecules, in an equilibrium with a negative heat and positive volume change of reaction.

The data in Tables I and II indicate that the pressure effect on the amount of precipitated protein is considerably less marked in the presence of alcohol, in some cases scarcely exceeding the range of experimental error.

With regard to the total reaction, the complexity of the kinetics makes it difficult to arrive at a satisfactory analysis until definitive data are available with respect to the possible significance of heterogeneity of the molecules and the specific reactions that are responsible for the results measured. Heterogeneity is perhaps an important factor in the apparent change in

rate of denaturation during the course of the reaction, thus making it desirable to use as homogeneous a preparation as possible in further studies. Apart from the initial lag, which was conspicuous at high pressures although hardly appreciable at normal pressure, straight lines with a slope of approximately 0.5 result when the logarithm of the percentage of the amount of protein in solution at the start is plotted against the logarithm of the time heated at 65°, with as well as without alcohol, under pressures up to

TABLE I

Influence of Temperature and Pressure on Amount of Precipitate Formed after Heating 0.686 Per Cent Globulin Solution for 40 Minutes at 65° and Atmospheric Pressure

Concentration of ethyl alcohol	Ppt. after standing 21 hrs. at room temperature		Ppt. after 21 hrs. at 4°		Ppt. after 21 hrs. at 4° under 10,000 lbs. pressure		Per cent increase in ppt. at 4° $\left(\frac{B-A}{A}\right)100$	Per cent decrease in ppt. at 4° under 10,000 lbs. pressure $\left(\frac{B-C}{B}\right)100$
	Per cc. protein in solution	Per cent pptd. (A)	Per cc. protein in solution	Per cent pptd. (B)	Per cc. protein in solution	Per cent pptd. (C)		
M	mg.		mg.		mg.			
0.0	3.58	47.8	2.96	57.0	3.30	51.9	19.3	8.9
0.2	2.42	64.8	2.09	69.6	2.27	67.0	7.4	3.7
0.3	2.04	70.3	1.70	75.2	1.94	71.7	7.0	4.7

TABLE II

Influence of 10,000 Pounds Pressure for 64 Hours at Room Temperature on Amount of Precipitate from 0.735 Per Cent Globulin Solution Treated 20 Minutes at 65° and Atmospheric Pressure

Concentration of alcohol	Per cc. protein in solution	Per cent pptd. (A)	After 64 hrs.				Per cent decrease in ppt. under pressure $\left(\frac{B-C}{B}\right)100$
			Atmospheric pressure		Under 10,000 lbs. pressure		
			Per cc. protein in solution	Per cent pptd. (B)	Per cc. protein in solution	Per cent pptd. (C)	
M	mg.		mg.		mg.		
0.0	4.40	40.1	4.30	41.5	5.28	28.6	31.1
0.2	3.12	57.5	3.10	57.8	3.55	51.7	10.5
0.3	2.58	64.9	2.53	65.6	2.87	61.0	7.0

5000 pounds per sq. in. Apparently the same relation holds for experiments with pressures of 10,000 pounds per sq. in., although fewer points are available along the curve, since the amount of denaturation was much less. The curves for the over-all reaction are thus largely the same except for the time scale, and the effect of pressure, as judged by these data obtained with the usual procedure, appears to be independent of the alcohol concentration. Furthermore, the magnitude of the pressure effect indicates a large volume increase of activation in the denaturation process,

of the order of 100 cc. per mole of protein, which is of the same order as the value calculated for the luminescent system either in the presence or absence of alcohol (4).

The authors take pleasure in acknowledging the interest as well as lengthy discussions and assistance of Professor Linus Pauling in connection with this study.

SUMMARY

At 65° and pH 6.0, the denaturation of highly purified human serum globulin at atmospheric pressure takes place at a complex rate which decreases progressively after about 40 per cent of the protein initially in solution has precipitated in the first 20 minutes. To some extent, the change in rate may be due to heterogeneity of the molecules. The specific rate, however, is very nearly the same with initial protein concentrations between 2.0 and 0.2 per cent.

The rate of denaturation is slowed by hydrostatic pressures up to 5000 pounds per sq. in., but the shape of the curve is the same, except for the difference in the time scale. A pressure of 10,000 pounds per sq. in. greatly retards the rate of precipitation and causes a marked initial lag of nearly an hour during which only slight precipitation occurs. The magnitude of the pressure effect indicates a volume increase of activation for the denaturation reaction of the order of 100 cc. per mole of protein in the process of activation of the reacting molecules.

The rate of denaturation at 65° is increased by ethyl alcohol, in relation to its concentration, from 0.05 to 0.60 M, but the shape of the curve remains essentially the same. Pressure retards the denaturation in the presence of alcohol, apparently independently of concentration up to 0.3 M.

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THE QUININE-OXIDIZING ENZYME AND LIVER ALDEHYDE OXIDASE*

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A derivative of quinine formed in minced rabbit liver has been isolated by Kelsey *et al.* (1), and identified by Mead and Koepfli (2) as a carbostyril. Hence quinine is oxidized in liver with replacement of the hydrogen atom in position 2 of the quinoline ring by a hydroxy group. Analogous oxidation products are excreted by men receiving the four principal cinchona alkaloids. This change is important in the chemotherapy of malaria, because the oxidation of these compounds markedly reduces their antimalarial activity (3). The activity of some quinoline compounds can be greatly enhanced if this oxidation is prevented by appropriate substitution (4). The nature of the metabolic system able to perform this oxidation is consequently of considerable pharmacological interest, and it was hoped that its biochemical function as well could be approached by studying the reaction with a series of quinoline derivatives. By the use of such compounds as substrates, the enzyme responsible for their oxidation has been prepared in about 5 per cent purity. It specifically acts on unsaturated heterocyclic compounds with an active α-hydrogen and is intimately associated with the flavoprotein, liver aldehyde oxidase.

Methods

Reagents—The synthetic antimalarials used were supplied by various laboratories cooperating with the malaria program of the Office of Scientific Research and Development. These "SN number" compounds are described fully elsewhere (4), but were generally of a high degree of purity. Isoquinoline, quinaldine, quinoline, and crotonaldehyde were redistilled *in vacuo*, and the bases recrystallized as the acid salts before use. Coenzyme I (5) and diaphorase (6) were prepared by the standard methods. Other compounds were used as obtained commercially. Propamidine was kindly furnished by Dr. D. F. Robertson of Merck and Company, Inc., and the pure quinine carbostyril by Dr. F. E. Kelsey.

Chemical Assays—For determination of the cinchona alkaloids the enzyme reaction was stopped with metaphosphoric acid, so as to eliminate blank fluorescence and adsorption of the drug on the precipitated proteins (7). Quinine carbostyril was determined in the resulting supernatant by its fluorescence at pH 10 in borate buffer, and compared with a standard in an identical blank preparation. The quinine fluorescence is completely quenched at this pH. Quinine was determined by its fluorescence in ethyl-
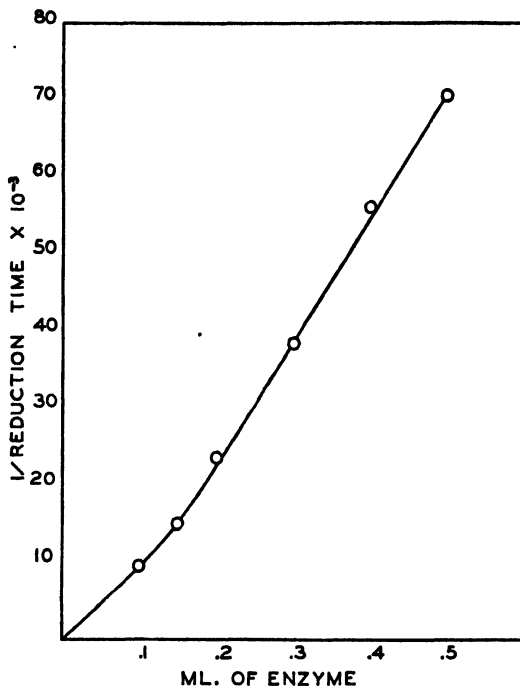
FIG. 1. Rate of cinchonidine oxidation as a function of enzyme concentration. Each Thunberg tube contained 0.3 ml. of 4×10^{-3} M cinchonidine, 0.1 ml. of 0.1 per cent methylene blue, and 0.1 M phosphate buffer, pH 7.6. Total volume 2.5 ml., 37°.

ene dichloride and trichloroacetic acid after removal of quinine carbostyril with NaOH by an unpublished method of Brodie. Cinchonidine was determined by the methyl orange method of Brodie and Udenfriend (8). The measurements of oxygen uptake were made in a Warburg manometer with the usual technique. The enzyme showed no blank oxygen uptake or CO_2 evolution. A Beckman spectrophotometer was used in the absorption determinations.

Assay of Enzyme—The activity of the enzyme is determined by the rate of methylene blue reduction with cinchonidine as substrate. A unit of enzyme

activity was arbitrarily chosen as that amount of enzyme in a total volume of 2.5 ml. which would reduce 0.1 ml. of 0.1 per cent methylene blue in 60 seconds. The reaction is carried out in phosphate buffer at pH 7.6 with a cinchonidine concentration above 2×10^{-4} M. The substrate is placed in the hollow stopper, the tube evacuated for 5 minutes, and the solutions mixed after 2 minutes incubation at 37°. Amounts of enzyme are chosen that will cause reduction in 20 to 60 seconds. During longer reaction periods the enzyme is progressively inactivated. The relation of enzyme concentration to the velocity of the reaction by this assay is shown in Fig. 1.

The destruction of the enzyme during activity occurs less rapidly in crude preparations. Thus liver brei will ·oxidize quinine aerobically at a linear rate for about 10 minutes, and more slowly for about an hour. The formation of quinine carbostyril in 10 minutes can therefore be used as an index of the activity of such preparations, provided an excess of quinine and oxygen is supplied. The two assays are not exactly comparable, however, since the enzyme reacts more slowly with oxygen than with methylene blue.

˙Preparation of Enzyme

Rabbit liver is the only satisfactory source of the enzyme yet found. No appreciable disappearance of added quinine was observed with breis of dog, pig, steer, sheep, or duck livers. Sheep liver brei can inactivate the enzyme of rabbit liver; possibly the enzyme in these other tissues is destroyed in the same way after mincing. This could not be avoided by the usual precautions of rapid manipulation at low temperatures.

The enzyme of rabbit liver was found to be soluble in water, to withstand heating to 60° for 5 minutes, and to be precipitated between 25 and 40 per cent saturation with ammonium sulfate. Successive application of these conditions yields about 5 ml. of a yellow-red solution from each 100 gm. of liver. This contains 20 units of the cinchonidine-oxidizing enzyme per ml. with about 4 mg. of protein per unit, compared to 26 mg. of protein per unit for the original brei. The enzyme is stable for over a month only if kept alkaline in solutions of ammoniacal ammonium sulfate (6 ml. of concentrated NH_3 per 100 ml. of saturated ammonium sulfate).

These purification steps were recognized as essentially similar to those used in preparing the flavoprotein, liver aldehyde oxidase, from pig liver (9). Other similarities were noted. Both enzymes are stable only in ammoniacal ammonium sulfate, both are rapidly destroyed during the catalytic process, and both are irreversibly inactivated by cyanide or by dialysis. And the preparation from rabbit liver does also have a strong aldehyde oxidase activity. Aldehyde oxidase prepared from rabbit liver, according to the

original method used by Gordon *et al.*, likewise oxidizes both aldehydes and quinolines, and is entirely similar in purity, properties, and activities to the first preparations of the quinoline-oxidizing enzyme. However, when aldehyde oxidase is prepared from pig liver by either method, no activity toward quinolines can be demonstrated.

The method of Gordon, Green, and Subrahmanyan has since been used for all preparations. This involves treatment of rabbit liver brei at 48° for 5 minutes in 25 per cent alcohol, lead acetate precipitation, and repeated fractionation with ammoniacal ammonium sulfate between 25 and 40 per cent saturation. A variety of other procedures were tried, which succeeded neither in furthering the purification nor in separating the two actions. These procedures included adsorption, dialysis, pH changes, freezing, drying, and precipitation with alcohol.

Characteristics of Enzyme

Since the agents responsible for quinoline and aldehyde oxidations could not be separated, both reactions have been studied for comparison. This enzyme oxidizes aldehydes to the corresponding acid with formation of equivalent amounts of hydrogen peroxide.

Reaction with Quinolines—The equation for the oxidation of quinine is

$$\text{Quinine} + H_2O + O_2 = \text{quinine carbostyril} + H_2O_2$$
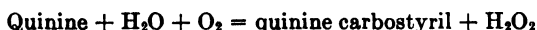
This was established by measurements of the reaction with both quinine and cinchonidine, compounds which differ only by the presence of a methoxy group in the former. Quinine, because of the ease with which its oxidation product can be determined, was used to show the equivalence of alkaloid oxidized to the carbostyril formed. Cinchonidine, being more soluble and more rapidly oxidized, was used in the measurements of oxygen uptake. The results are shown in Table I. It will be noted that the molar ratio of oxygen absorbed to cinchonidine removed is 0.6, instead of 1.0 as expected if H_2O_2 is a product. This is due to liberation of oxygen from some of the H_2O_2, a reaction referable to the catalase present as an impurity in the enzyme preparation. Keilin and Hartree's observation (10) that hydrogen peroxide can oxidize alcohol in the presence of catalase makes it possible to prove that H_2O_2 is formed during the oxidation simply by adding ethyl alcohol. This is shown in the last two experiments of Table I. The oxygen released by the fission of H_2O_2 was removed in this way and the molar ratio of oxygen taken up to cinchonidine oxidized is seen to approximate 1.0. No oxygen uptake occurs with the enzyme and alcohol alone.

That oxidation occurs only in the 2 position of the quinoline ring was further confirmed by identifying the product of the oxidation of quinoline itself. After overnight aeration with 30 ml. of enzyme, 200 mg. of quinoline

were shown by spectrophotometric analysis to be quantitatively converted to a compound giving the absorption curve of carbostyril. Quinoline treated similarly with heat-inactivated enzyme was unchanged. The carbostyril was isolated in asbestos-like crystals by ether extraction and precipitation from 0.1 N NaOH with CO_2, and recrystallized from alcohol and water. It was sparingly soluble in water, gave no color with ferric chloride, formed a barium salt crystallizing in leaflets, and melted at 196–197°. The mixed melting point with carbostyril (m.p. 196–197°) was unchanged (temperatures uncorrected).

TABLE I

Balance of Quinine or Cinchonidine Disappearance with Quinine Carbostyril Formation and Oxygen Uptake

Warburg vessels containing 0.4 ml. of enzyme, total volume 3.0 ml.; air in gas space; 37°. Quinine experiments, incubated 1 hour with 1.0 ml. of saturated quinine, 0.1 M phosphate buffer, pH 7.2. Cinchonidine experiments, incubated 20 minutes with 1.5 ml. of 4×10^{-3} M cinchonidine, 0.1 M phosphate buffer, pH 7.5; the last two experiments also contained 0.1 ml. of ethyl alcohol.

Substrate	Substrate dis- appearance	Quinine carbostyril formed	Oxygen uptake	$\dfrac{\text{Quinine carbostyril}}{\text{Quinine}}$	$\dfrac{O_2}{\text{Cinchonidine}}$
	M	M	M		
Quinine	1.42	1.32		0.93	
"	1.43	1.30		0.91	
Cinchonidine	2.57		1.69		0.66
"	3.23		2.06		0.62
"	2.65		1.73		0.65
" + alcohol	1.99		1.97		0.99
" + "	1.86		1.72		0.93

Substrate Concentration—The maximum rate of cinchonidine oxidation at pH 7.6 occurs with concentrations of 2×10^{-4} M or higher. By the use of one-fifth the usual concentration of methylene blue, a rate approximately half the maximum was observed with an initial cinchonidine concentration of 0.5×10^{-4} M. Quinine at pH 7 shows a maximum rate with concentrations above 5.4×10^{-4} M and a half maximum rate at about 1.7×10^{-4} M (Fig. 2). Cinchonine and quinidine react maximally only at the somewhat higher concentration of 8×10^{-4} M.

The optimum substrate concentration for crotonaldehyde oxidation at pH 8.3 is 0.060 M or higher. A half maximum rate occurs with 0.017 M crotonaldehyde (Fig. 3).

Optimum pH—The rates of cinchonidine and quinine oxidations at different pH values in phosphate buffers are seen in Fig. 4. In borate buffers the rates of these oxidations are about 50 per cent less.
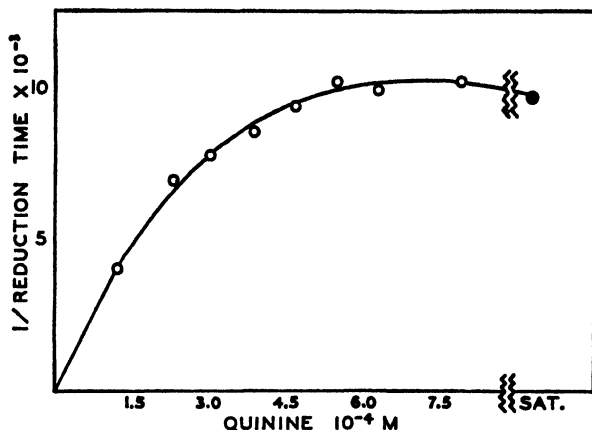
FIG. 2. Effect of quinine concentration on the rate of its oxidation. Each tube contained 0.3 ml. of enzyme, 0.1 ml. of 0.1 per cent methylene blue, 0.06 M phosphate buffer, and various amounts of 1.5×10^{-3} M quinine in buffer. Total volume 2.4 ml., 37°. ⊗ = saturated by the addition of crystalline quinine.
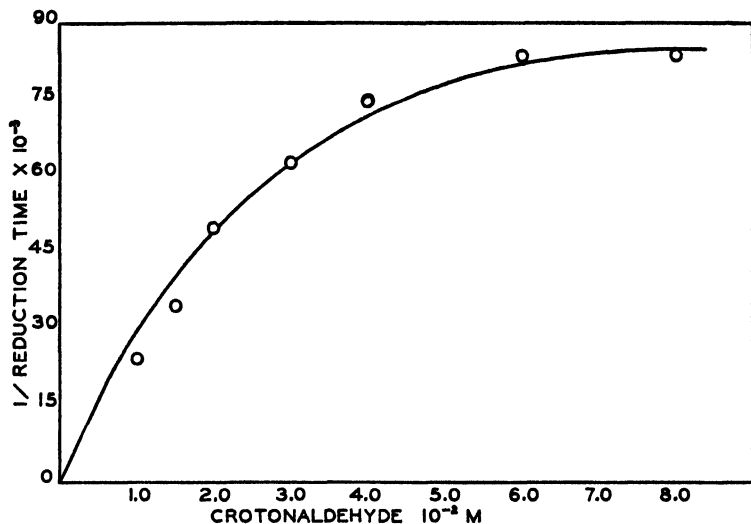
FIG. 3. Effect of crotonaldehyde concentration on the rate of its oxidation. Each tube contained 0.3 ml. of enzyme, 0.1 ml. of 0.1 per cent methylene blue, 0.1 M borate buffer, pH 8.3, and various amounts of 0.5 M crotonaldehyde. Total volume 2.5 ml., 37°.

The rate of aldehyde oxidation is optimum at pH 8.2 to 8.6, and is the same in borate or phosphate buffers.

Specificity—It is apparent from the data in Table II that many com-

pounds with an α-hydrogen in a quinoline, isoquinoline, or substituted pyridine ring can be oxidized.   This oxidation is prevented by substitution of a phenyl or carboxyl group for the α-hydrogen.   Quinaldine, which has an α-methyl group, is oxidized, but here too the methyl hydrogen atoms are nearly as active as the quinoline α-hydrogen.   The rate of oxidation is affected by substituents elsewhere in the structure that would be expected to alter the activity of the α-hydrogen.   Thus a 6-methoxy group decreases
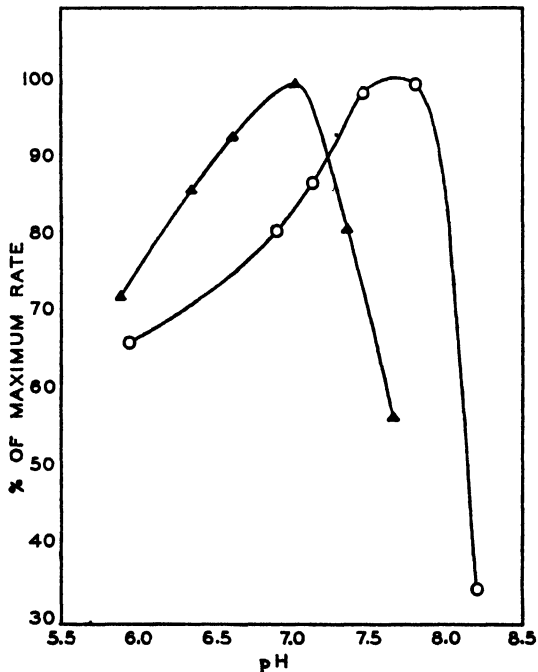
FIG. 4. Rates of quinine (▲) and cinchonidine (O) oxidations as a function of pH. In each tube 0.3 ml. of enzyme, 0.1 ml. of 0.1 per cent methylene blue, 0.15 M phosphate buffer, and either 0.3 ml. of 4 × 10⁻³ M cinchonidine or 1.0 ml. of saturated quinine hydrochloride.   Total volume 2.5 ml., 37°.   The final pH was determined with the glass electrode.

the rate of oxidation in the cinchona alkaloids and in the simpler quinolines. This also occurs with chloro and even more with dichloro substitution.   The 8-aminoquinoline, plasmochin, is not oxidized at all.   Alkylation of the pyridine or quinoline nitrogen, which increases the activity of the α-hydrogen, in both N¹-methylnicotinamide and quinoline ethiodide also increases the rate of oxidation markedly.

The slower oxidation of methoxy-, chloro-, and amino-substituted quinolines suggests that the enhanced antimalarial effect of quinolines

possessing such groups may be due in part to a decreased rate of enzymic inactivation in the body. The rate of this oxidation is apparently deter-

TABLE II

Specificity of Quinoline-Oxidizing Enzyme

Thunberg experiments, in each tube 6 units or less of enzyme, 0.1 M phosphate buffer, pH 7 5, 0 1 ml. of 0 1 per cent methylene blue, and 2.4×10^{-3} M substrate; total volume 2.5 ml ; 37°. Values are in per cent of the cinchonidine rate under the same conditions. Reduction times longer than 1 hour are expressed as $(+)$. Controls without substrate or without enzyme were not reduced in 48 hours.

Substrate	Relative rate
Cinchonidine .	100
Quinine	15
Cinchonine	7
Quinidine	0 6
SN2549, 4-α-(2-piperidyl)quinoline methanol	143
SN8539, SN2549 with 7-chloroquinoline	62
SN2157, SN2549 " 6-methoxyquinoline	42
SN10956, SN2549 " 6-methoxy-2-methylquinoline .	$(+)$
SN8534, SN2549 " 6-chloroquinoline	41
SN10276, SN2549 " 8-chloroquinoline	49
SN10278, SN2549 " 6,8-dichloroquinoline	25
SN8538, SN2549 " 2-phenylquinoline	0
SN7618, 6-chloro-4-(diethylaminomethylbutyl)aminoquinoline	$(+)$
SN7135, SN7618 with 2-methylquinoline	$(+)$
Quinoline	51
Isoquinoline	53
Quinoline ethiodide	160
4-Methylquinoline (lepidine)	37
4-Carboxyquinoline (cinchoninic acid)	0
6-Methoxyquinoline	0
8-Aminoquinoline	2.5
8-Amino-6-methoxyquinoline	1 0
8-Hydroxyquinoline .	$(+)$
Quinaldine	31
4-Chloroquinoline	31
4,7-Dichloroquinoline	$(+)$
4-Hydroxyquinoline-2-carboxylic acid (kynurenic acid)	0
4,8-Dihydroxyquinoline-2-carboxylic acid (xanthurenic acid)	0
Nicotinic acid and amide	$(+)$
N^1-Methylnicotinamide	37
Coenzyme I	$(+)$
Dihydrocoenzyme I	$(+)$

mined by the α-hydrogen, while the antimalarial effect persists even in the absence of this group.

The action of the enzyme on N^1-methylnicotinamide raised the pos-

sibility that it might also function on the pyridine nucleotide coenzymes somewhat as does diaphorase. Though no information is available about any enzyme similar to this one in the malaria parasite, such a mechanism might explain the action of quinine. However, the enzyme does not oxidize coenzyme I or dihydrocoenzyme I at a significant rate. Furthermore, quinine and cinchonidine do not inhibit the action of diaphorase. Haas observed a similar lack of specific inhibition from quinine with dihydrocoenzyme II oxidation by cytochrome reductase (11).

The failure of the enzyme to oxidize other structurally suitable compounds can generally be correlated with the absence or inactivity of the α-hydrogen atom in a heterocyclic ring. Various other heterocyclic and aromatic compounds besides those listed were tested. Thus the acridine (atabrine), purine (adenine and hypoxanthine), imidazole (histamine), and benzene (l-tyrosine) ring systems are not oxidized. Indole itself, but not $l(-)$-tryptophane, and pyrrole, but not N-ethylpyrrole, are slowly oxidized. The enzyme does not oxidize pyridine, α-picoline, piperidine, pyridoxine, or arecoline.

The same enzyme preparations acting on the above compounds also oxidize crotonaldehyde and benzaldehyde in the anaerobic system respectively at 166 and 90 per cent of the rate of cinchonidine oxidation. Acetaldehyde is rapidly oxidized but its volatility precludes accurate measurement of its rate by this method. The ratio of quinoline and aldehyde oxidation rates did not change during the later stages of purification of the enzyme and was the same in all preparations. No other oxidative enzymic functions were found in the purified preparations. Occasionally a preparation showed traces of xanthine oxidase or l-amino acid oxidase activity, but this was removed by the ammonium sulfate fractionation.

Inhibition—In Table III it will be seen that equal inhibitions of both aldehyde and quinoline oxidations are produced by cyanide, propamidine, and plasmochin. A saturated solution of caprylic alcohol likewise inhibits both activities. The inhibition of cinchonidine oxidation by 8-hydroxyquinoline, which is itself slowly oxidized, suggests that plasmochin too may inhibit by combining specifically with the enzyme. The failure of the slowly oxidized pyridine derivatives to inhibit cinchonidine oxidation probably reflects their lower affinity for the enzyme.

Simultaneous Oxidation of Quinolines and Aldehydes—No summation of the rate of either oxygen uptake or of methylene blue reduction occurs when the enzyme acts simultaneously on both types of substrate. The rate is often slower than with either substrate alone. The interference is well shown by manometric experiments in which both oxygen uptake and cinchonidine disappearance were measured. Cinchonidine in a concentration of 2×10^{-4} M had an initial rate of oxygen uptake of 12.1 microliters

per 2 minutes, and during the 20 minute experiment 1.73 micromoles of O_2 and 2.64 micromoles of cinchonidine reacted. Crotonaldehyde alone caused a similar rate of oxygen uptake. However, in the presence of 0.07 M crotonaldehyde as well as cinchonidine, the initial rate of oxygen uptake fell to 3.5 microliters per 2 minutes, and only 0.72 micromole of O_2 and 0.28 micromole of cinchonidine reacted. Similar interference with the anaerobic oxidation of cinchonidine is produced by the presence of aldehyde.

TABLE III

Inhibitors of Cinchonidine and Crotonaldehyde Oxidation

Thunberg experiments, 0.3 ml. of enzyme, 0.1 M phosphate buffer, pH 7.5, 0.1 ml. of 0.1 per cent methylene blue; total volume 2.5 ml.; substrate and methylene blue in hollow stopper, inhibitor with enzyme in tube; incubated 2 minutes at 37° before mixing.

Inhibitor		Substrate		Inhibition
	M		*M*	*per cent*
Cyanide	1×10^{-2}	Cinchonidine	4.8×10^{-4}	97
"	1×10^{-2}	Crotonaldehyde	8×10^{-2}	97
Propamidine	4×10^{-4}	Cinchonidine	4.8×10^{-4}	50
"	4×10^{-4}	Crotonaldehyde	8×10^{-2}	82
Plasmochin	4×10^{-4}	Cinchonidine	4.8×10^{-4}	45
"	8×10^{-4}	Crotonaldehyde	8×10^{-2}	41
8-Hydroxyquinoline	2.4×10^{-3}	Cinchonidine	4.8×10^{-4}	87
SN7618	8×10^{-4}	"	4.8×10^{-4}	0
Atabrine	2×10^{-3}	"	4.8×10^{-4}	60
Nicotinamide .	2.4×10^{-3}	"	4.8×10^{-4}	0
N^1-Methylnicotin-amide	2.4×10^{-3}	"	4.8×10^{-4}	0
Noval diamine	2.4×10^{-3}	"	4.8×10^{-4}	5
SN8538	2.4×10^{-3}	"	4.8×10^{-4}	0

Reversal of Quinine Oxidation—The mutual interference of the two reactions can be attributed partly to a dismutation as well as to competition for one enzyme. Thus, in an anaerobic system containing aldehyde the enzyme will reduce some quinine carbostyril to quinine. With 1 ml. of enzyme, 99 γ of quinine carbostyril, and 0.4 ml. of 0.5 M crotonaldehyde mixed anaerobically and incubated 20 minutes, amounts of 0.9 to 1.1 γ of quinine are formed. This amount, though small, is readily determined by the fluorometric method. The quinine was further identified by its solubility in ethylene dichloride at different pH values and by its characteristic pH-fluorescence curve.

Nature of Prosthetic Group—Like the aldehyde oxidase of pig liver, the enzyme of rabbit liver is also a flavoprotein. The flavin is easily demonstrated after splitting the purified preparation by heating at 70° and pH

3.8 for 5 minutes. The yellow supernatant shows the typical absorption curve of a flavin nucleotide with maxima at 265, 373, and 451 mμ. It was identified as flavin adenine dinucleotide by use of the split d-amino acid oxidase system (12). All the flavin present is in this form, since assays by this method or by the total absorption at 451 mμ agree within the experimental error. Calculated as riboflavin, the enzyme contains 1.1 γ per unit of aldehyde oxidase activity, in the units used by Gordon and coworkers. In their purified aldehyde oxidase from pig liver, these earlier workers also found 1.1 γ of riboflavin per unit.

Quantitative reduction of this flavin in the intact enzyme by aldehyde could not be demonstrated, since the absorption at 451 mμ referable to flavin is only 10 per cent of the total light absorption. However, addition of acetaldehyde to the enzyme in the absence of oxygen decreases the absorption at this wave-length approximately as expected from the specific reduction of the flavin group. In the same way, cinchonidine similarly decreases the absorption at 451 mμ. And since H_2O_2 is formed during the oxidation of cinchonidine, it is most likely that the same flavin also acts as the prosthetic group or as a hydrogen acceptor in this reaction.

The enzyme contains 0.7 γ of riboflavin per unit of cinchonidine activity. Assuming one flavin group per molecule, the turnover number is 150 for the enzyme acting on cinchonidine in the anaerobic system. If a molecular weight of 70,000 is assumed for this enzyme in common with other flavoproteins, a preparation which contained 0.05 per cent riboflavin and 1.3 mg. of protein per unit is calculated to be about 5 to 10 per cent pure.

DISCUSSION

The aldehyde oxidase of rabbit liver is identified with that of pig liver by their similar characteristics and equal amounts of riboflavin per unit. Consequently the association of the aldehyde oxidase with the quinoline-oxidizing enzyme in rabbit liver and its absence in pig liver are difficult to explain. Yet the similarity of the two enzymes in rabbit liver is striking. They possess similar characteristics in regard to solubility, heat resistance, adsorption, stability in ammoniacal ammonium sulfate, and lability on dialysis and acidification. They also show approximately the same degree of activity, the same inhibitions, and undergo a similar destruction during activity. There is a difference in pH optima, which is perhaps explicable in terms of the decreased solubility of the quinoline compounds at alkaline pH, and a difference in the effect of borate buffers as yet unexplained. These similarities and the mutual interference of the two substrates suggest that the two reactions must share at least one element in the oxidation pathway. The evidence seems to indicate that one element both reactions share is the flavin group.

The quinoline oxidation cannot be simply a unique extension of the aldehyde oxidase specificity. The oxidation of quinoline may well be a dehydrogenation of the dihydro-α-hydroxy derivative formed by the addition of water to the nitrogen and migration of the hydroxy group to the α position (13). Aldehyde oxidation is also thought to be a dehydrogenation reaction. However, if the same active groups that oxidize aldehydes also oxidized quinolines, the very active aldehyde oxidase of pig liver should oxidize quinolines. This inactivity of the enzyme from pig liver can best be explained by the destruction of the separate active groups for quinoline upon mincing the tissue. The association of the two actions, at least in rabbit liver, may therefore result from a common protein moiety. This would be very similar to the association of both aldehyde and purine oxidations with xanthine oxidase.

The reversal of quinine oxidation by coupling it with aldehyde oxidation also suggests that this is an enzyme with two different sets of active groups and a single head, the flavin prosthetic group. A similar view of the other dual function enzyme, xanthine oxidase, is held, since Booth (14) has demonstrated that the anaerobic oxidation of salicylaldehyde by xanthine oxidase will reduce uric acid to hypoxanthine.

As yet a readily acceptable substrate for the quinoline-oxidizing function in metabolism is lacking. In view of the demonstrated specificity of the enzyme for the α position, its failure to oxidize kynurenic and xanthurenic acids was to be expected, although these are the only known quinoline compounds occurring physiologically. If a reaction similar to the oxidation of quinolines occurs physiologically, the substrate must presumably possess an oxidizable α-hydrogen in a heterocyclic ring. N^1-Methylnicotinamide, which reacts at least 70 times as rapidly as nicotinamide itself, may well undergo this oxidation in the body. The product of this oxidation, which would be expected to be the α-pyridone derivative, does not fluoresce under the conditions used to determine the parent compound (15). This oxidation may therefore account for the disappearance of N^1-methylnicotinamide administered to men that was observed by Perlzweig and Huff (16).

SUMMARY

1. The enzyme of rabbit liver oxidizing quinine and other quinolines to their carbostyrils has been prepared in about 5 per cent purity. The solution contains 0.7 γ of riboflavin per unit of activity. The flavin is reduced anaerobically by cinchonidine. Aerobically H_2O_2 is formed in the reaction.

2. The enzyme has a pH optimum between 7.4 and 7.6, and reacts with cinchonidine concentrations above 2×10^{-4} M at a maximum rate. It reacts directly with oxygen but more rapidly with methylene blue, and is rapidly destroyed during catalysis.

3. The oxidation of quinolines is limited to the α position and the rate of oxidation is correlated with the activity of the α-hydrogen. Quinolines, isoquinoline, and some pyridine derivatives are oxidized.

4. The physiological compound most rapidly oxidized is N^1-methylnicotinamide.

5. The enzyme has properties similar to, ‘and is associated with the flavoprotein, liver aldehyde oxidase. All the flavoprotein present is accounted for by the aldehyde oxidase. The simultaneous oxidation of aldehyde and quinoline is slower than their separate rates. The anaerobic oxidation of aldehyde will reduce the quinine carbostyril to quinine.

6. The enzyme is considered to be a flavoprotein with two functions like those of xanthine oxidase.

The flavin adenine dinucleotide assays were kindly made by Dr. P. K. Stumpf. Dr. G. L. Hobby of Chas. Pfizer and Company, Inc., and Dr. Herbert Stoerk were most helpful in providing rabbit livers.

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A MODIFIED PROCEDURE FOR THE PREPARATION OF PROTOPORPHYRIN IX DIMETHYL ESTER FROM HEMOGLOBIN*

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Protoporphyrin IX dimethyl ester, a crystalline compound with a definite melting point, is generally used to identify protoporphyrin IX. Various methods of preparation (1–4) have been described in the literature. As indicated by Grinstein and Watson (5, 6), application of chromatographic separation is necessary in order to obtain a pure product. By following the procedures thus far described in the literature the same conclusion has been reached. However, by modifying the procedure as heretofore described, we have been able to obtain the product in a pure state without the necessity of applying chromatographic separation.

A solution of crude protoporphyrin IX was prepared from defibrinated blood by Hamsik's procedure (7) with some modifications. Either citric acid or salicylic acid can be used in the place of oxalic acid. The solution of crude protoporphyrin was then successively extracted with ether and 10 per cent hydrochloric acid and converted into the dimethyl ester, as described below. For crystallization of the ester it has been found that a mixture of acetone and ether is more satisfactory than chloroform and methanol.

This procedure has been successfully employed to prepare crystalline protoporphyrin IX dimethyl ester from a sample of red blood cells as small as 0.15 ml. In the case of small samples, the amounts of materials used are proportionally reduced and the time of reaction shortened.

EXPERIMENTAL

A sample of 5 ml. of red blood cells, after being separated from the plasma by centrifuging, was thoroughly stirred with 50 ml. of acetone. The precipitate was collected on a sintered glass filter and then refluxed for 15 to 20 minutes with 100 ml. of acetone solution containing 10 gm. of oxalic acid. The reaction mixture was cooled and mixed successively with 5 gm. of stannous chloride and then 20 ml. of concentrated hydrochloric acid. It was allowed to stand at room temperature for 1 to 1½ hours. A saturated solution of sodium acetate was then added until the purplish

red solution turned to a brownish red.   With addition of 5 ml. of glacial acetic acid and filtration (or centrifugation) the supernatant solution or filtrate was extracted three times with 20 ml. of ether.   In case there was no separation of two layers at this point, more ether was added.   The ether solution was extracted with 10 ml. of 10 per cent hydrochloric acid three to four times.   The 10 per cent hydrochloric acid extract (also containing some acetone and ether) was mixed with a saturated solution of sodium acetate until a brownish red color resulted.   The ether layer was separated off and washed with a little water.   The extract was again extracted with 5 ml. of 10 per cent hydrochloric acid three times.   The combined 10 per cent hydrochloric acid extract was mixed with an equal volume of methanol previously saturated with hydrogen chloride, and allowed to stand at room temperature for 2 to 3 hours.   Then a saturated solution of sodium acetate was added until the solution became brownish red.   It was extracted with chloroform twice.   The chloroform extract was washed with 10 per cent ammonium hydroxide, 7 per cent sodium chloride, and a little distilled water.   The filtered chloroform solution was distilled on a steam bath to remove the solvent.   The residue was stirred with a mixture of acetone and ether (1:2) and centrifuged.   The dark red prisms thus obtained melted at 223°; yield 5 to 7 mg. of protoporphyrin IX dimethyl ester per ml. of red blood cells.

Thus far it has been found that 10 per cent hydrochloric acid solution is the best medium for esterification.   Either 5 or 25 per cent hydrochloric acid solution gave unsatisfactory results.

SUMMARY

A modified method of preparing pure protoporphyrin IX dimethyl ester from hemoglobin, without application of chromatographic separation, has been described.   It has been applied successfully to micro as well as macro samples of red blood cells.

For crystallization of the ester a mixture of acetone and ether (1:2) is more satisfactory than chloroform and methanol.

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THE EFFECT OF HYPOPHYSECTOMY AND ADRENO-CORTICOTROPIC HORMONE ON THE ALKALINE PHOSPHATASE OF RAT PLASMA*

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Effect of Hypophysectomy

It is well known that the phosphatase content of bone, kidney, liver, and blood may change under varying conditions of growth, diet, and disease (1). Among these tissues, blood perhaps furnishes the over-all changes of this enzyme activity. Weil (2) found a rise in plasma phosphatase activity from birth to maturity during the growth of normal rats. Kinard and Chanutin (3) have reported that the phosphatase content of the whole rat increases from the time of birth. There is ample · evidence that certain bone diseases cause a marked alteration in plasma phosphatase (4). Furthermore, phosphatase has been shown to play a part in bone regeneration (5).

The effects of hypophysectomy on bone histology have been described recently by Becks, Simpson, and Evans (6). The immediate reaction is a thinning of the cartilage plate, and after longer postoperative intervals the bony trabeculae become coarser and less numerous. Since the phosphatase activity is intimately related to changes in bone, one may expect that changes may occur in plasma phosphatase after hypophysectomy. In a survey of the literature, there appears only one report (7) on serum phosphatase activity in hypophysectomized rats.

EXPERIMENTAL

Male rats used were of the Long-Evans strain. Hypophysectomy was performed at 40 days of age by the parapharyngeal approach. The completeness of the operation was ascertained at autopsy by examination of the sella turcica. The animals were maintained on the usual diet of this laboratory *ad libitum*. Blood was taken from the inferior vena cava after the animals were anesthetized by the intraperitoneal administration of sodium amytal. 4 per cent sodium citrate solution (0.5 cc. per sample) was used as the anticoagulative agent. The alkaline phosphatase in the plasma was determined immediately or within 24 hours. The plasma samples were always kept frozen at $-15°$.

The alkaline phosphatase activity was estimated by a modification of the method of King and Armstrong as described by Binkley, Shank, and Hoagland (8); at least three determinations were carried out on each plasma sample. The Cenco-Sheard-Sanford photelometer with orange filter was employed for recording the color intensity.

The micro-Kjeldahl method was used for nitrogen determination. The phosphatase potency was expressed either in units[1] per 100 mg. of nitrogen or per 100 cc. of plasma.

Plasma Phosphatase Content of Normal Male Rats—Results on the plasma alkaline phosphatase activity of male rats of different ages are summarized in Table I. It will be noted that the phosphatase activity increases from 21 to 40 days of age and remains practically constant up

TABLE I

Phosphatase Content in Plasma of Male Rats at Different Ages

Age	No. of rats	Body weight	N per cc. plasma	Phosphatase	
				Per 100 mg. N	Per 100 cc. plasma
days		*gm.*	*mg.*	*units*	*units*
21	10	47.5 ± 1.65*	7.00 ± 0.28	4.17 ± 0.45	29.2
40	11	142.0 ± 3.07	7.71 ± 0.14	5.67 ± 0.45	43.8
40	10†	126.3 ± 3.50	7.94 ± 0.11	4.89 ± 0.35	38.8
55	10	211.5 ± 6.64	8.43 ± 0.11	4.90 ± 0.40	41.3
90	10	257.6 ± 5.94	9.56 ± 0.17	4.52 ± 0.61	43.3

* Mean ± standard deviation.

† Fasting 48 hours.

to 90 days of age when the enzyme activity is expressed on the basis of 100 cc. of plasma. When the phosphatase potency is expressed in units per 100 mg. of N, the activity seems to decline after 40 days of age.[2] It has been recorded by Weil (2) that "the high plasma phosphatase activity of the rat 1 month old decreases gradually with the maturity of rat, but remains above the original low enzyme value." The same conclusion was also arrived at by Gould (9).

It is of interest to note that the nitrogen concentration in the plasma increases gradually from 7.0 mg. to 9.56 mg. per cc. as the rats grow from

[1] A unit of alkaline phosphatase is that amount of activity which will liberate 1 mg. of phenol in 30 minutes in barbiturate buffer of pH 9.7.

[2] From nine rats 7 days of age with an average body weight of 17.0 gm., we have obtained the plasma alkaline phosphatase activity of 4.75 units per 100 mg. of nitrogen. The nitrogen content of the plasma is 6.2 mg. per cc. These values were secured from one sample of pooled plasma. It appears that the enzyme activity of these young rats seems to be lower than that of the 40 day-old animals.

21 days to 90 days. The latter value agrees with that reported by Levin (10) who found that serum in adult male rats eating *ad libitum* contains 6.04 per cent protein or 9.66 mg. of nitrogen per cc.

The effect of fasting on the plasma phosphatase has been studied by Weil and Russell (11) and Gould (9). They found that normal rats show a decrease of enzyme activity after fasting for 24 hours. However, Gould's experiments with fat-fed animals indicated that 1 day of fasting caused little change in the serum phosphatase level. As shown in Table I, there is a definite lowering of plasma alkaline phosphatase after 48 hours of fasting, but less marked than that observed by Weil and Gould.

Plasma Phosphatase Content of Hypophysectomized Male Rats—From Table II, it is evident that hypophysectomy decreases the alkaline phos-

TABLE II

Phosphatase Content in Plasma of Hypophysectomized Male Rats at Different Postoperative Periods

40 days old at operation.

Post-opera-tive	No. of rats .	Body weight		N per cc. plasma	Phosphatase	
		Initial	Final		Per 100 mg. N	Per 100 cc. plasma
days		*gm.*	*gm.*	*mg.*	*units*	*units*
0	11	142.0 ± 3.10*		7.71 ± 0.14	5.67 ± 0.45	43.8
4	10	144.7 ± 3.12	130.6 ± 2.82	8.77 ± 0.16	4.23 ± 0.31	37.1
8	10	154.5 ± 4.45	133.3 ± 3.88	8.96 ± 0.24	3.45 ± 0.34	30.9
15	17	147.1 ± 1.98	124.6 ± 2.02	8.67 ± 0.16	3.46 ± 0.22	30.0

* Mean ± standard deviation.

phatase activity of male rat plasma. In the 4th day after the operation, the phosphatase level changes from 43.8 to 37.1 units per 100 cc. of plasma. The enzyme level continues to fall but becomes constant after 8 days after hypophysectomy.

It is very well known that hypophysectomy causes atrophy of the adrenal, thyroid, and reproductive organs as well as the cessation of body growth. Therefore, the influence of hypophysectomy on the plasma phosphatase may be attributable to over-all changes in the function of the adrenal, thyroid, and reproductive organs as well as to the cessation of body growth. For instance, a lowering of serum phosphatase has been observed by Watson (12) after injection of adrenal cortical extract and an increase of the enzyme upon the administration of testosterone propionate (13). Moreover, thyroxine is known to increase the phosphatase content of the bone (14). Since, as already mentioned, rapidly growing rats have a higher phosphatase

level in the serum, it may be expected that a loss of body weight may produce a lowering of the enzyme activity. The depression of alkaline phosphatase in male rat plasma after hypophysectomy may therefore be explained by the loss of growth, on lessening in function of thyroid or gonad, or the combination of these factors. On the other hand, from the lowering in adrenal function due to the removal of the pituitary one would expect an elevation of the plasma phosphatase level. It is clear from the data just presented that the influence of lessened adrenal function is completely overshadowed by the effect from the other deficiences which result from hypophysectomy.

It is of interest to compare our results with those obtained by Jones and Shinowara (7). They found that hypophysectomy causes an elevation of serum phosphatase activity. Their observations were made with female

TABLE III

Phosphatase Content in Plasma of Normal and Hypophysectomized Female Rats

Type of animal	No. of animals	Body weight	Phosphatase per 100 mg. N
		gm.	*units*
Normal*	10	106.6 ± 2.6	6.05 ± 0.40
Hypophysectomy	18†	74.1 ± 1.5	3.01 ± 0.38
	17‡	69.8 ± 2.3	3.11 ± 0.30

* 41 to 42 days old.
† Operation at 26 to 28 days of age; 11 to 18 days postoperative.
‡ Operation at 26 to 28 days of age; 20 to 24 days postoperative.

rats. It was thought that the disagreements between our findings and theirs may be due to the sex difference of the animals employed. We have therefore investigated the alkaline phosphatase content of hypophysectomized *female* rats as compared to their normal female controls.

Table III summarizes the results obtained with normal and hypophysectomized female rats. The animals were operated upon when 26 to 28 days of age; two groups of different postoperative periods were used: 11 to 18 days and 20 to 24 days. No evident difference was observed in the phosphatase level between these two groups of hypophysectomized rats. The normal animals employed were 41 to 42 days old and their plasma phosphatase activity served for comparison. It is clear that, as found in male rats, the plasma alkaline phosphatase content in hypophysectomized female rats is definitely lower than that in normal animals.

Effect of Adrenocorticotropic Hormone

The relationship of the adrenals to phosphatase in animal tissues has been studied by a number of investigators. Williams and Watson (15)

have shown that corticosterone reduces the phosphatase content in rat femurs, while desoxycorticosterone acetate produces an increase. In an earlier paper Watson (12) reported that adrenal cortical extract lowers the serum phosphatase but desoxycorticosterone acetate has no effect. Furthermore, adrenalectomy produces a marked lowering of phosphatase in the cat kidney (16). It would appear of considerable interest to investigate the plasma alkaline phosphatase level in animals with adrenals hypertrophied by the administration of adrenocorticotropic hormone.

In Table IV, results obtained with hypophysectomized male rats are summarized. The adrenocorticotropic hormone was isolated from sheep pituitaries by the method previously described (17). Male rats hypophysectomized at 40 days of age were injected intraperitoneally immediately

TABLE IV

Effect of Adrenocorticotropic Hormone on Phosphatase Content in Plasma of Hypophysectomized Male Rats

| Experiment on 17 rats | Body weight | | Adrenals | N per cc. plasma | Phosphatase | |
	Initial	Final			Per 100 mg. N	Per 100 cc. plasma
	gm.	*gm.*	*mg.*	*mg.*	*units*	*units*
Injected*..	144.6 ± 2.52†	112.7 ± 2.49	25.0 ± 1.10	9.63 ± 0.24	2.61 ± 0.24	25.1
Control....	147.1 ± 1.98	124.6 ± 2.02	10.5 ± 0.45	8.67 ± 0.16	3.46 ± 0.22	30.0

* Rats hypophysectomized at 40 days of age; injections with 0.20 mg. of hormone daily began on the day of operation once daily for 15 days except Sundays; *i.e.*, thirteen injections in 15 days.

† Mean ± standard deviation.

after operation with 0.2 mg. of adrenocorticotropic hormone daily (except Sunday) for 15 days. As was shown in an earlier report (17), this daily dose is sufficient to maintain the weight of adrenals at 25.0 mg., while the hypophysectomized controls have adrenal weights of 10.5 mg. The data indicate that, when alkaline phosphatase is expressed either in units per 100 mg. of N or per 100 cc. of plasma, the enzyme level is significantly lower in the hormone-treated group than in the control.

Similar experiments were carried out in 40 day-old normal male rats. A total daily dose of 1.0 mg. of adrenocorticotropic hormone was employed; intraperitoneal injections were instituted three times daily, twice on Saturday, and once on Sunday for 15 days. The results in Table V show that the averaged adrenal weights of six injected animals were 50.3 mg., while those of the controls were 29.3 mg. The increase of adrenal function is further displayed by both the inhibition of body growth and the

reduction of thymus weight in the injected group as compared with the controls. The alkaline phosphatase content in the plasma is convincingly reduced by the hormone; the hormone-treated animal has an average of 28.4 units of phosphatase per 100 cc. of plasma, whereas the untreated male rats of 55 days of age possess 41.3 units.

From bone studies, it was found that adrenocorticotropic hormone caused a retardation in both chondrogenesis and osteogenesis in the region of the proximal epiphysis of the tibia of normal rats. (18). Moreover, the inhibiting action of adrenocorticotropic hormone on the growth of young and adult male rats has been clearly demonstrated (19); it may therefore be concluded that the reduction of alkaline plasma phosphatase activity by the hormone is probably due to the lowering of the enzyme content in the

TABLE V

Effect of Adrenocorticotropic Hormone on Phosphatase Content in Plasma of Normal Male Rats

Experiment	No. of rats	Body weight		Adrenals	Thymus	N per cc. plasma	Phosphatase	
		Initial	Final				Per 100 mg. N	Per 100 cc. plasma
		gm.	*gm.*	*mg.*	*mg.*	*mg.*	*units*	*units*
Injected*	6	130.7 ±5.83†	164.1 ±3.08	50.3 ±2.91	123.8 ±11.75	9.02 ±0.34	3.15 ±0.15	28.4
Control	10	142.0 ±3.07	211.5 ±6.64	29.3 ±0.85	410.1 ±10.23	8.43 ±0.11	4.90 ±0.40	41.3

* 40 day-old male rats; 1.0 mg. of hormone divided into three injections daily. On Saturday two injections and once only on Sunday, for 15 days.

† Mean ± standard deviation.

osseous tissues. To verify this presumption data on tibia phosphatase activity should be known. Such studies are planned for future investigations.

A comparison of the results herein reported with those obtained by Watson (12, 15) when adrenal cortical hormones were employed seems to indicate that the adrenals hypertrophied by adrenocorticotropic hormone secrete mainly, if not wholly, steroids which have an oxygen atom on C_{11}, rather than substances akin to desoxycorticosterone. This deduction is in agreement with the conclusion of Fraenkel-Conrat *et al.* (20) from studies of liver arginase.

SUMMARY

The alkaline phosphatase in the plasma of male rats at various ages (21 to 90 days) has been determined. After hypophysectomy, the enzyme

activity in the plasma of male rats 40 days of age decreases with successive postoperative periods (4 to 15 days). A similar lowering of the plasma phosphatase values in female rats occurs after removal of the pituitary.

In normal and hypophysectomized male rats the administration ˙of adrenocorticotropic hormone in doses causing hypertrophy of the adrenals produced a significant decrease in the alkaline phosphatase level of the plasma.

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THE ESTIMATION OF LYSOZYME BY A VISCOSIMETRIC METHOD*

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Hitherto the bacteriolytic action of lysozyme has been assayed by measuring the clearing of suspensions of organisms exposed to the agent. The susceptibility of test organisms exposed to lysozyme is variable. These variations are due to enzymes contained within the bacterial cells. In a previous publication (1) it was pointed out that concomitantly with the lysis by lysozyme there appears a large amount of non-protein N and inorganic phosphate. Paralleling this breakdown process, many enzyme activities of the intact or acetone-killed organisms are found to disappear completely when the organisms are laked with lysozyme. From this it is evident that bacterial lysis is a complex reaction which involves the participation of the bacterial enzymes as well as of lysozyme.

A test of lysozyme activity, based on a single chemical reaction, was obviously desirable. The mechanism of lysozyme activity has been explained in terms of the hydrolysis of a substance of mucoid nature contained within the bacterial wall. Such mucoid material was extracted from the cells and was found to be hydrolyzed by lysozyme, as was shown by the liberation of reducing substances (1). These observations were confirmed and partly extended by other investigators (2, 3). However, this reaction was rather slow compared to the speed of bacterial lysis, apparently because of the impurity and degraded state of the substrates. The isolation of the substrates of lysozyme in their high polymer form was finally achieved. The depolymerization of this material, as measured viscosimetrically, proved to be a specific, accurate, and speedy test for lysozyme activity.

EXPERIMENTAL

The isolation of the substrate of lysozyme in a native form involved considerable difficulty. The organisms most susceptible to lysozyme, *viz.* *Micrococcus lysodeikticus* and some strains of *Sarcina lutea*, are highly resistant to mechanical disintegration. Even ultrasonic treatment leaves the cells intact (Dr. P. K. Stumpf).[1] Cells ground in a number of bacterial

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[1] Private communication.

mills yielded no carbohydrate on extraction with alkaline salt solutions. From these and other experiments it must be concluded that the mucoid exists within the bacterial wall as an insoluble complex. In earlier work the mucoid was extracted by 2.5 N sodium hydroxide at 100° (1), by treatment with formamide at 170°, or by treatment with cold hypochlorite followed by hot formamide solution (2). None of the fractions obtained from these extracts exhibited any appreciable viscosity, nor was the mucoid present in any reasonable state of purity. The use of alkali, which is known to be highly destructive to many mucopolysaccharides, could not be avoided, since no method was found of bringing the substrate into solution other than the use of lysozyme. When heavy suspensions of the microorganisms were exposed to lysozyme for a few minutes, followed by the inactivation of the enzyme by iodine, no viscous carbohydrate was obtained. (Such solutions have yielded a viscous solution whose viscosity could be abolished by desoxyribonuclease, a sample of which was obtained from Dr. M. McCarty (4). This sample was free of lysozyme but, when added to suspensions of organisms together with lysozyme, a greater clearing was effected than by lysozyme alone.) Extraction with 90 per cent phenol left the organisms intact. The preparation of a viscous mucopolysaccharide fraction was finally made possible by prolonged treatment with 0.5 N sodium hydroxide at 25°.

Method of Preparation of Crude Mucopolysaccharide Fraction

Micrococcus lysodeikticus was grown for 48 hours in Roux bottles on Difco nutrient agar enriched with 0.5 per cent glucose and 0.1 per cent yeast extract. Almost double the yield of organisms was obtained on a medium consisting of Difco heart infusion agar fortified with 0.5 per cent glucose. (However, the isolation of viscous fractions from organisms grown in this medium proved to be much more difficult.) The organisms were washed off the agar with cold water, filtered through a "milk pad" (Schwartz Manufacturing Company) to remove larger agar particles, and poured into 5 volumes of ice-cold acetone. After 24 hours at 0° the organisms were collected by filtration through a Büchner funnel, washed with acetone and ether, and dried *in vacuo*.

For extraction 10 to 15 gm. of dried organisms were ground to a fine paste in 200 cc. of 0.5 N sodium hydroxide and kept in an atmosphere of nitrogen for 7 to 8 days at 25°. The suspension was then diluted with 200 cc. of water and the cells separated by sharp centrifugation. The supernatant fluid contains practically none of the substrate of lysozyme, but a good deal of inert material, including carbohydrates derived from agar. The packed cells were washed with water, again separated by centrifugation, resuspended in 200 cc. of 0.5 N sodium hydroxide, and kept in nitrogen at about

25° for another 14 days. The cells were disintegrated by that time. The viscous material was diluted with 200 cc. of water and cleared by centrifugation. The residue, after being washed, was not cleared by incubation with lysozyme, nor did it yield any reducing substances. The alkaline supernatant solution was acidified to about pH 4 with glacial acetic acid and poured into 2 volumes of alcohol. The fibrous precipitate was collected by centrifugation and washed repeatedly with 80 per cent alcohol. The moist alcoholic material was dissolved in 200 cc. of 10 per cent sodium acetate, diluted with 200 cc. of water, stirred for 1 hour with 80 cc. of chloroform-amyl alcohol mixture, and finally centrifuged. To the turbid supernatant solution 40 cc. of 10 per cent zinc acetate were added and the pH adjusted to about 7.0 until flocculation of the zinc hydroxide occurred. The material was cleared by centrifugation, and the supernatant solution acidified to about pH 4 and precipitated with 2 volumes of alcohol. The precipitate was collected by centrifugation, washed repeatedly in a mortar successively with 80 per cent, 95 per cent, and absolute alcohol and ether, and dried *in vacuo* over P_2O_5. The yield varied between 1.5 and 2 gm. for 10 gm. of dried bacteria.

From a strain of *Sarcina lutea*, isolated from the laboratory air, a similar carbohydrate fraction has been isolated. This strain of *Sarcina lutea* is laked with egg white lysozyme at about the same dilution as is needed for lysis of *Micrococcus lysodeikticus*.[2] The crude polysaccharide fraction of this organism, in contrast to the fraction from *Micrococcus lysodeikticus*, contains in addition to the substrate of lysozyme a carbohydrate which forms a viscous solution but is not attacked by lysozyme.

From a third organism, *Staphylococcus muscae*, a similar carbohydrate has been obtained, though in much lower yield. This *Staphylococcus* requires for lysis a concentration of lysozyme some 30 times greater than that required for the two previously mentioned organisms.

The crude *Micrococcus lysodeikticus* fractions[3] contain between 5.5 and 6.5 per cent of nitrogen and 23 to 30 per cent of hexosamine (5). On incubation of these fractions (5 mg. per cc.) with lysozyme (0.04 to 0.1 mg. per cc.) for 2 hours, about 10 per cent of the weight appears as reducing sugar, expressed as equivalents of glucose. About one-half of this value can be determined as acetylglucosamine (6).

Lysozyme Preparations—The egg white lysozyme used in these experiments was prepared either by the method used in this laboratory (7) or by

adsorption on bentonite (8). One sample of crystalline lysozyme was obtained from the Western Regional Laboratory.

Viscosimetrio Method—In the viscosimetric method the carbohydrate fraction is dissolved to give a 0.4 per cent solution in McIlvaine's buffer (9) of pH 5.3, containing as a rule 0.2 M NaCl. The viscosity of these solutions remains constant for about 24 hours, provided they are kept in the ice box. At 37° the viscosity slowly decreases, probably owing to oxidative depolymerization. However, this spontaneous loss of viscosity is insignificant
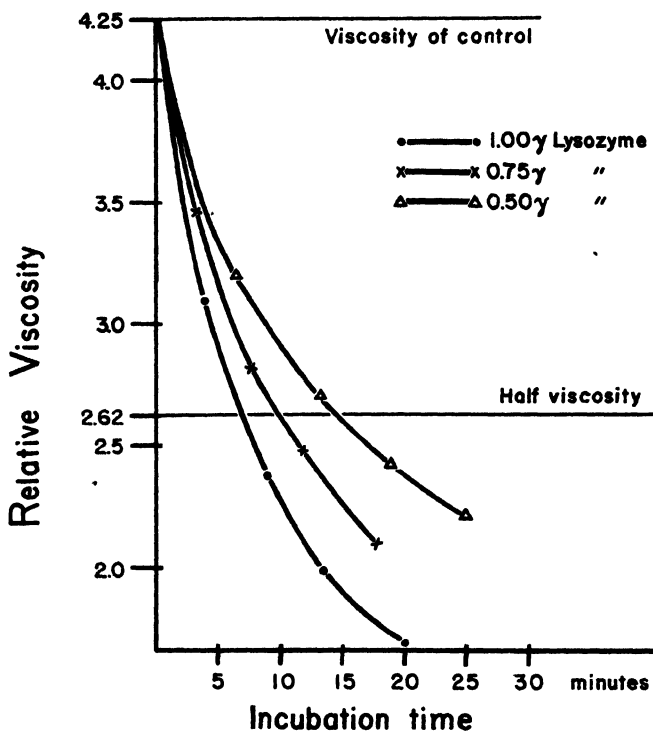
Fig. 1. Influence of lysozyme concentration on rate of depolymerization of mucopolysaccharide.

if lysozyme concentrations are chosen which catalyze half the reaction within 30 minutes.

The substrate solution (5.0 cc.) is warmed to 37° in a constant temperature bath and mixed with 1.0 cc. of lysozyme present in an appropriate concentration in 0.9 per cent sodium chloride. The time of mixing is taken as zero time. 5 cc. of the mixture are transferred immediately to an Ostwald viscosimeter having a flow time of about 66 seconds for 0.9 per cent sodium chloride. At least four successive readings are made on each sample. The viscosity of the control solution (0.9 per cent sodium chloride

without lysozyme) is measured repeatedly. The viscosity of the control solutions varies somewhat with different preparations, but its value is usually about 4. With the carbohydrate fractions prepared from *Micrococcus lysodeikticus*, the time necessary to reach half viscosity corresponds closely to the calculated value, since the relative viscosity after depolymerization with lysozyme drops to very close to unity. With the carbohydrate fractions prepared from *Sarcina* there remains a considerable

TABLE I

Viscosimetric Estimation of Activity of Crystalline and Amorphous Lysozyme of Egg White

Substrate No.	Lysozyme No.	Lysozyme	Half time	Lysozyme containing 1 unit	Date tested
		γ	min.	γ	1945
20D	W. R.*	1.0	7.5	0.75	Dec. 26
20D	"	1.0	7.5	0.75	" 21
20D	"	1.0	7.0	0.70	" 6
20D	"	1.0	7.5	0.75	Nov. 29
20C	"	1.0	7.5	0.75	Dec. 4
6I	"	1.0	7.2	0.72	Nov. 29
20C	X85C	1.0	9.5	0.95	" 27
20C	X85C	2.0	4.5	0.90	" 27
20C	M17	1.0	7.0	0.70	" 27
20C	M17	1.0	7.0	0.70	Dec. 6
					1946
35CII	110CI	1.0	7.5	0.75	Jan. 21
35CII	110CI	1.0	7.5	0.75	" 26
35CII	113A	1.0	15.0	1.50	" 21
35CII	113A	1.0	15.0	1.50	" 26
35CII	113D	1.0	8.0	0.80	" 21
35CII	113D	1.0	8.3	0.83	" 25
35CII	113DII	1.0	6.5	0.65	" 21
35CII	113DII	1.0	7.0	0.70	" 25

*Western Regional Research Laboratory.

viscosity after the action of lysozyme, but if this residual viscosity is subtracted, the values for the half time of lysozyme activity are the same as those of the *Micrococcus lysodeikticus* fractions.

The relative viscosities are plotted on graph paper against the time of incubation. 1 unit is defined as the amount of enzyme required to reach half viscosity in 10 minutes. With crystalline egg white lysozyme 1 unit is contained in about 0.73 γ. The time required to reach half viscosity is proportional to the enzyme concentration. This is illustrated in Fig. 1. In this experiment 1.00 γ of lysozyme required 7.0 minutes to reach half viscosity, 0.75 γ required 10.0 minutes, and 0.50 γ required 14.5 minutes. The accuracy and reproducibility of the method are evident from Table I

in which it can be seen that the error is about ±5 per cent. This error is mostly due to the timing of mixing and to the graphical extrapolation used. By increasing the accuracy of both procedures, the error probably could be still further decreased. Table I further shows that some of the preparations made by our method are as pure as the crystalline material of the California group. The high electrophoretic mobility of our preparation in borate solution (10) is apparently due to complex formation with the borate.[4]
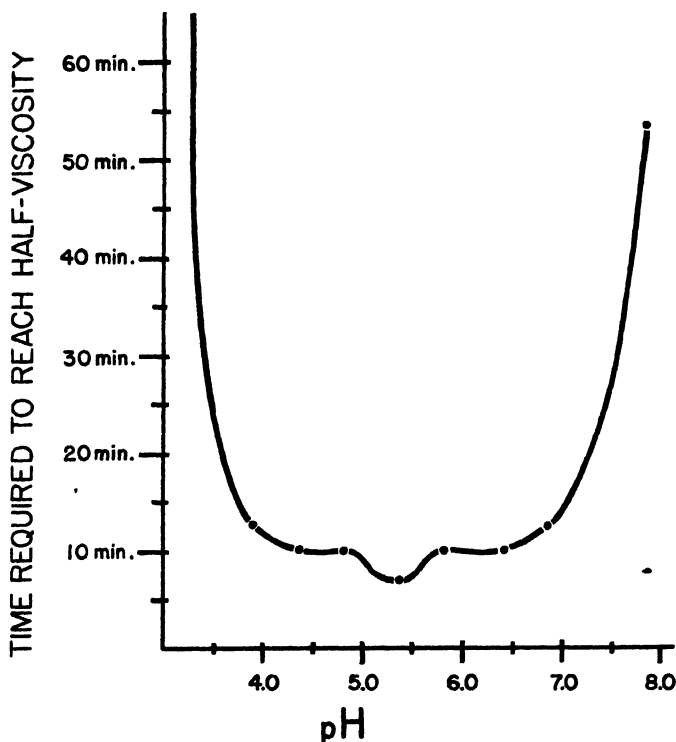
FIG. 2. Influence of pH on lysozyme activity as measured viscosimetrically

[4] The activity of the reaction is not increased by biotin. The biotin effect previously reported (10) is observed only with a labile variant strain of *Micrococcus lysodeikticus*, which was characterized by a remarkable tendency to autolyze. Avidin preparations tested in this laboratory both by the bacteriolytic and viscosimetric methods had lysozyme activity in all cases. The lysozyme activity was highest with the avidin preparations of greatest antibiotin potency. The preparations of avidin included material prepared according to Woolley and Longsworth (11) and according to the California group (12). Our lysozyme preparations were devoid of avidin activity. It still appears to us most probable that egg white lysozyme and avidin are related to each other and that one protein is derived from the other. The plant lysozymes (13), however, do not occur together with an agent acting as antibiotin.

Previously the pH optimum for egg white lysozyme, as determined by measuring the rate of increase of reducing sugar, was given as about pH 3.5 (1). The pH curve as determined by the viscosimetric method is shown in Fig. 2. In this experiment, samples of substrate were prepared in McIlvaine's buffer of different pH levels and 1 γ of egg white lysozyme was added to each solution. Controls without lysozyme were measured at each pH, as the viscosity varied somewhat with changes in pH. It can be
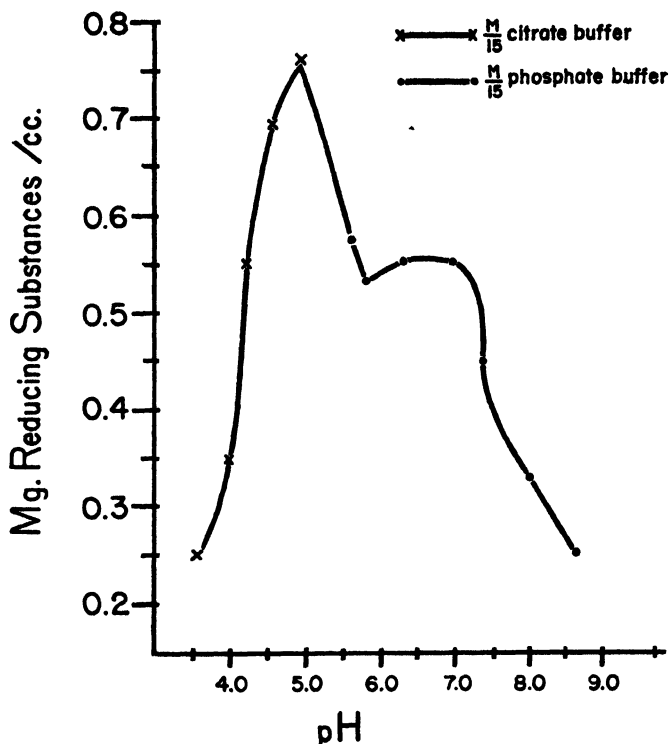
Fig. 3. Influence of pH on lysozyme activity as measured by the increase in reducing groups.

seen that the optimum is at pH 5.3, as measured by the glass electrode. The pH dependence curve, as measured by the increase in reducing sugar, is shown in Fig. 3. Samples of substrate (10 mg. per cc.) were incubated with egg white lysozyme (0.04 mg. per cc.) in $M/15$ buffers. After 20 hours the solutions were titrated by the ceric sulfate method (14) and the pH measured by the glass electrode. Optimum rate of hydrolysis is observed at pH 4.95.

The addition of 0.2 M sodium chloride had no influence on the increase in reducing sugar. However, if lysozyme activity is measured viscosi-
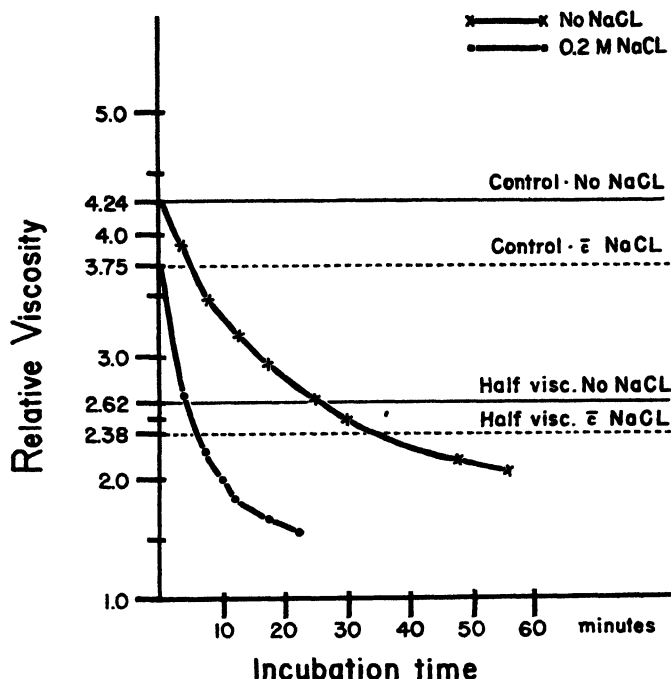
FIG. 4. Influence of sodium chloride on lysozyme activity as measured viscosimetrically.

TABLE II

Viscosimetric Estimation of Lysozyme Activity of Some Fluids and Tissues

Source	Dilution tested	Half time	Lysozyme units per cc. or gm. original
		min.	
Egg white	1:10,000	8.0	12,500
Tears	1:500	5.5	950
	1:1,000	10.5	
Saliva H	1:1	15.0	1.3
" M	1:1	10.5	1.9
" F	1:1	11.5	1.7
Guinea pig cartilage (rib)	1:10	10.5	10.0
" " serum	1:5	60 (Approximate)	0.8 (Approximate)
" " colon	1:5	100 "	0.5 "
" " tear gland	1:10	200 "	0.5 "
Human cartilage (rib)	1:20	5.5	36.3

metrically, addition of sodium chloride leads to a remarkable increase in activity, as can be seen from Fig. 4, in which 1 γ of lysozyme was added to the substrate solution with and without salt.

Applications of Method—The concentration of lysozyme in native egg white, in a sample of pooled tears, in filtered saliva, in serum and some tissue extracts of a guinea pig was determined viscosimetrically and the results are shown in Table II. The tears (stimulated by onions) were collected from three normal adults and cleared by centrifuging. On the basis of total protein the lysozyme titer of tears is somewhat higher than that of egg white. The cartilage and tear glands of the guinea pig were ground with sand and 0.9 per cent sodium chloride and the mixture was centrifuged. The colon was rinsed thoroughly with water and its mucosa scraped off and ground with sand. The human cartilage was obtained at autopsy of a 14 months old baby. The actual titer may be still considerably higher, since after grinding many large particles remained and since no effort was made to elute adsorbed lysozyme from the sand. The high lysozyme titer of the cartilage in comparison with the other tissues seems remarkable.

DISCUSSION

The viscosimetric method for the determination of lysozyme seems far superior to the bacteriolytic methods in use now. In accordance with other workers, we have found that the sensitivity of living organisms to lysozyme varies greatly with changes in media and strains. In our experience this is likewise true for organisms killed by various methods. The explanation for this variation apparently lies in changes in the autolytic enzymes contained within the bacterial cell, which contribute to a varying degree to the visible clearing of bacterial suspensions.

Lysozyme has many characteristics of another mucolytic enzyme, hyaluronidase. Like the latter, it is reversibly oxidized, and in low concentrations it primarily catalyzes the depolymerization of its substrate with little or no apparent increase in reducing groups. Furthermore, in both enzyme systems, chloride ion greatly increases the depolymerase action, while the opening of glucosidic linkages does not seem to require halogen.[5] The chloride effect, in our experiments, is more pronounced with hyaluronidase than with lysozyme, presumably because less chloride was present in the preparations. It seems that these mucolytic enzymes in common with some other polysaccharidases like amylase and pectinase can function as either depolymerase or glucosidase, depending on whether the enzyme is combined with chloride. It remains to be seen whether the chloride is bound by a metal or by a specific organic group within the protein molecule.

One of the most important problems is the function of lysozyme in various fluids and cells. Its occurrence in microbes, including those sensitive to the enzyme (15), and in tissues like cartilage suggests that its function is primarily metabolic, concerned with the depolymerization or hy-

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[5] Meyer, K., and Hahnel, E., unpublished.

drolysis of its substrate. Its rôle as a defense mechanism then would be purely incidental. In the case of hyaluronidase, a similar multiplicity of function has been established. Hyaluronidase can protect against streptococcal infection (16), testicular hyaluronidase is concerned with mammalian reproduction (17), while different bacterial hyaluronidases may facilitate infection in making available to the microorganisms carbohydrates essential to their growth. If the function of lysozyme is considered to be primarily metabolic, the wide occurrence of the substrate is to be anticipated. However, thus far a search for the substrate of lysozyme in extracts of various organs has been futile.

SUMMARY

1. A method has been described for the isolation from a number of microorganisms of a highly polymerized mucopolysaccharide fraction which can serve as the specific substrate of lysozyme.

2. This polysaccharide is depolymerized by lysozyme obtained from different sources. With larger concentrations of lysozyme, glucosidic linkages are hydrolyzed and reducing sugar and acetylhexosamine appear.

3. The depolymerization of this mucopolysaccharide, as measured viscosimetrically, has been used as the basis of an accurate and rapid method of lysozyme determination.

4. Some data are given for the lysozyme titers in different tissues.

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LYSOZYME OF PLANT ORIGIN*

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Our knowledge of lysozyme, the bacteriolytic and mucolytic enzyme found in egg white and in many tissues and body fluids, is almost entirely based on the properties of the enzyme obtained from egg white. The wide distribution of an agent which is bacteriolytic for certain micrococci, *Sarcinae*, and other air-borne organisms, was recognized by Fleming who also reported its occurrence in some plants, especially the turnip (1).

The occurrence of a lysozyme-like enzyme in samples of papain was detected when it was observed that the high viscosity of a solution containing a mucopolysaccharide fraction isolated from *Micrococcus lysodeikticus* disappeared on incubation with papain-HCN. Bacteriological tests showed the presence of a lytic enzyme in the papain preparation used. Other samples of papain showed similar activities. Subsequently large concentrations of such lytic enzymes were found in a crude preparation of ficin, a material obtained from the latex of Central American *Ficus* trees (*Ficus glabatra* and *Ficus doliana* (2)).

Like their equivalent in egg white, the plant enzymes lake a number of air organisms. They depolymerize a mucopolysaccharide fraction prepared from such organisms, and hydrolyze this fraction with the liberation of acetylhexosamine. The plant lysozymes, however, differ from egg white lysozyme in that they have a more limited range of antibacterial activity. Furthermore, the *Ficus* lysozyme, which was investigated more thoroughly than the papain lysozyme, is chemically clearly distinct from the enzyme prepared from egg white.

EXPERIMENTAL

The mucolytic activity was determined as described in the preceding paper (3). The bacteriolytic activity was measured with *Micrococcus lysodeikticus* as the test organism. 24 to 48 hour agar slant cultures were suspended in primary M/15 phosphate solution and the suspension was appropriately diluted to match barium sulfate standard No. 10 (4). 0.5 cc. of this suspension was incubated for 1 hour at 37° with 0.5 cc. of lysozyme solution in 2-fold serial dilutions in primary phosphate. Then 2 drops

of N sodium hydroxide were added and the clearing of the suspensions was read visually by comparison with a control. Units are expressed as the highest dilution giving complete visible clearing under these conditions.

Papain—The papain samples tested included a crude commercial material (Merck), crude and purified commercial material obtained several years ago from Japan, samples purified by precipitation with alcohol (5) including one sample kindly sent us by Dr. Joseph Fruton, preparations by the lead and aluminum hydroxide method of Stumpf and Green,[1] and fractions obtained by bentonite adsorption and elution with 5 per cent pyridine sulfate (6).

Table I summarizes data on the mucolytic activity of some papain samples. The activity compared to the values for ficin is low in all in-

TABLE I

Lysozyme Activity of Papain Preparations As Measured Viscosimetrically

Preparation No.	Lysozyme	Buffer
	units per mg.	
MII26*	1.9	0.1 M acetate, pH 5.0
Fruton's†	4.0	0.1 " " " 5.0
26BIII‡	6.5	0.1 " " " 5.0
22A§	5.0	McIlvaine's, pH 5.3
Sumeritin‖	4.5	" " 5.3

* Prepared by adsorption on bentonite.
† Prepared by alcohol fractionation and obtained from Dr. J. Fruton.
‡ Prepared from crude papain by alcohol fractionation.
§ Prepared according to Stumpf.
‖ Commercial purified Japanese sample.

stances. The bacteriolytic activity of the papain preparations likewise indicated a very low titer. As with most preparations complete lysis was not observed, even with the highest concentrations tested, it is difficult to assign numerical values to the bacteriolytic tests. (Many of the papain samples became turbid on the addition of alkali.) All the papain samples showed lysis with *Micrococcus lysodeikticus* and *Sarcina*. With *Staphylococcus muscae*, lysis was apparent only in the presence of a reducing agent.

The low lysozyme titer of papain is likewise evident from the data on hydrolytic activity summarized in Table II, which represent the increase in reducing sugar found after incubation with papain preparations. Under comparable conditions egg white lysozyme hydrolyzed 10 per cent of the weight of the polysaccharide in 2 hours, whereas papain liberated at most

[1] Stumpf, P. K., and Green, D., personal communication.

3.8 per cent in 23 hours. From other experiments it can be assumed that the lysozyme concentration in the experiment with egg white lysozyme was at least 10 times the required amount.

Ficin—The ficin preparations used included a sample of crude ficin.[2] This material was purified either by the lead acetate fractionation used for papain[1] or by adsorption on bentonite. In the latter procedure 20 gm. of the crude material were dissolved in 200 cc. of 1 per cent potassium chloride and acidified to about pH 4 with N hydrochloric acid, an insoluble residue was removed by centrifugation, and a suspension of 10 gm. of bentonite in 100 cc. of 1 per cent potassium chloride was added with vigorous stirring. Stirring was continued for 5 minutes and the suspension centrifuged. About 50 per cent of the activity remained in the supernatant solution.

TABLE II

Hydrolysis of Mucopolysaccharide

Mixture in M/7.5 citrate buffer, pH 4.95	Hydrolysis	
	2 hrs.	23 hrs.
	per cent	*per cent*
Substrate (10 mg. per cc.) + Sumeritin S (2 mg. per cc.)	2.0	3.8
Substrate (10 mg. per cc.) + crude papain (2 mg. per cc.)	1.3	2.5
Substrate (10 mg. per cc.) + Papain 2 (2 mg. per cc.)	1.5	3.5
Substrate (10 mg. per cc.) + Papain 4 (2 mg. per cc.)	1.5	2.5
Substrate (10 mg. per cc.) + lysozyme W. R.* (2 mg. per cc.)	10.0	10.0

* Western Regional Research Laboratory.

The adsorbed material was eluted three times with 0.2 M phosphate of pH 7.5. The supernatant solutions were centrifuged, filtered through a sintered glass filter, and dialyzed first against cold running tap water, then against distilled water at 4°, allowed to stand in the cold for several days, clarified by centrifuging, and lyophilized. These preparations, and those obtained from the fractionation with sodium chloride, as will be seen from Table III, were the most potent.

From neutral solution, little ficin was adsorbed by bentonite. In con-

trast to egg white lysozyme, all activity adsorbed can be eluted with phosphate. Practically no further activity is recovered with 5 per cent pyridine or pyridine sulfate solution.

TABLE III

Comparison of Bacteriolytic, Mucolytic, and Proteolytic Titer of Samples of Ficus Preparations

Preparation	Bacteriolytic titer		Mucolytic titer		Proteolytic titer
	Organisms			pH	
		units per mg.	*units per mg.*		*units per mg.*
Egg white lysozyme*	*Micrococcus lysodeikticus*	260	1370	5.3	Negative
	Sarcina lutea	260			
	Staphylococcus muscae	16			
Crude ficin†	*M. lysodeikticus*	64	714	3.5	31.0
	S. lutea	64	333	5.3	
	St. muscae	1 (Approximate)			
Ficin 13*	*M. lysodeikticus*	Negative	0.4 (Approximate)	3.5	100.0
" 14*	" "	<0.5	2.6	3.5	71.4
" 25A‡	" "	130	1000	3.5	
	S. lutea	130			
" 29AI§	*M. lysodeikticus*	130	1100	5.3	14.3
" 29AII§	" "	16– 30	27	5.3	33.3
" 29B‖	" "	16	24	5.3	22.2
" 29G¶	" "	32– 64	500	3.5	
" 29H¶	" "	64–130	2000	3.5	
" 29J¶	" "	64	1335	3.5	
" 29K¶	" "	64–130	2000	3.5	
" 29L¶	" "	130	1670	3.5	11.1
" 32AI§	" "		2220	3.5	8.4
" 32AII§	" "	64	2860	3.5	7.7

* Crystalline.
† Merck and Company, Inc.
‡ Lead acetate preparation.
§ Adsorption on bentonite and elution with phosphate.
‖ Adsorption on bentonite and elution with pyridine and pyridine sulfate.
¶ Sodium chloride fractionation.

Table III illustrates the correlation between the bacteriolytic titer, as determined by the visible clearing of live organisms, and the mucolytic titer as determined viscosimetrically. Considering the inaccuracy of the bacteriolytic method and the fact that the fractions were tested with many different batches of organisms, the correlation appears to be satisfactory. It seems to be especially significant that the two samples of crystalline

ficin (7),[3] which had a high proteolytic activity, were almost completely devoid of lysozyme activity in both tests.

It was evident from the data on the adsorption of ficin on bentonite (and from electrophoretic data) that the physical properties of the *Ficus* lysozyme are distinctly different from those of egg white. This is likewise indicated in Fig. 1, in which the mucolytic activity of a ficin preparation was determined at different pH levels with McIlvaine's buffer. As the viscosity of the control varied with the pH, the control viscosity at each
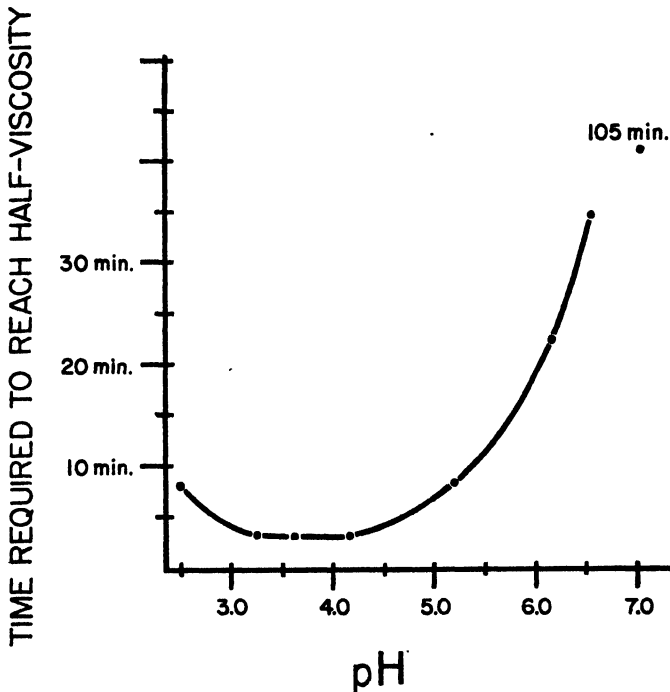
FIG. 1. Effect of pH on activity of *Ficus* lysozyme, as measured viscosimetrically

pH was determined. The pH optimum with *Ficus* lysozyme lies between pH 3.2 and 4.2. The optimum pH for egg white lysozyme is at pH 5.3 (3). In accordance with the low activity of the *Ficus* lysozyme at neutral pH, there was likewise only a trace of activity at neutrality in the bacteriolytic test. This shows the importance of carrying out the bacteriolytic test at acid reaction, followed by alkalization at the end of the incubation period.

Egg white and *Ficus* lysozyme also differ with respect to stability. Crystalline egg white lysozyme and a *Ficus* preparation each in concentration

of 100 γ per cc. in McIlvaine's buffer of pH 5.0 and 8.0 were heated for 10 minutes at 100°. These were then diluted and tested viscosimetrically against the same substrate. With egg white lysozyme the unheated control contained 1 unit in 0.75 γ, while the samples heated at pH 5 and 8 contained 1 unit in 1.7 and 140 γ respectively. These results show the great stability of this enzyme in acid solution and the markedly greater lability at an alkaline pH. With the *Ficus* lysozyme, the unheated control contained 1 unit in 1 γ. Both heated samples were completely negative in concentrations of 100 γ per cc.

Egg white and *Ficus* lysozyme also differ in their behavior towards some strains of *Micrococcus lysodeikticus* and *Sarcinae*. A strain of *Micrococcus lysodeikticus* was adapted to egg white lysozyme by growing the organisms in increasing concentrations of egg white lysozyme. An adapted strain

TABLE IV

Milk-Clotting Activity of Lysozyme Preparations

Sample	Clotting time
No. MII22A, papain, 800 γ + SO₃	101.9
" " " 80 " + "	979
Ficin, Merck, 800 γ + SO₃	30
" " 80 " + "	440
" " 800 " no "	30
W. R.,* lysozyme, 800 γ + SO₃	Negative, discontinued after 24 hrs.
No. 97C, lysozyme, 800 γ + SO₃	" " " 1½ "

* Western Regional Research Laboratory.

which required 16 times the concentration of egg white lysozyme for complete lysis had become completely refractory to *Ficus* lysozyme. Experiments are in progress to determine whether the adaptation is due to destruction of the lysozymes. Strains naturally resistant to egg white lysozyme likewise arc less sensitive to or are not affected by *Ficus* lysozyme.

It seemed of special importance to determine whether the proteolytic activities of papain and ficin are in any way related to the bacteriolytic activity. The data reported in Tables III and IV rule out the possibility of any correlation. Proteolytic activity of some preparations was measured by the milk-clotting power (*cf.* Table IV), with a commercial dry milk powder as a substrate.[4] 20 gm. of Dryco were triturated with 100 cc. of 0.1 N acetate buffer of pH 5 until homogeneous. The fat was separated by centrifuging and removed. Enzyme solution (0.1 cc.) and 0.005 per cent sodium sulfite (0.1 cc.) or 0.9 per cent sodium chloride (0.1 cc.) were pipetted

[4] We thank Dr. Paul Stumpf for information about this test.

into a tube. After 5 minutes at 37°, 2 cc. of the milk, previously warmed to 37°, were blown into the tube and the clotting time observed. A control tube without enzyme was similarly prepared. It can be seen from Table IV that crude ficin had a much higher proteolytic activity than did papain. Two different samples of egg white lysozyme failed to show any activity in clotting milk. Egg white lysozyme, furthermore, in a concentration of 2 mg. per cc. after incubation for 24 hours with the polysaccharide fraction (20 mg. per cc.) gave no increase in amino nitrogen (Van Slyke).

In Table III the proteolytic activities of some *Ficus* samples are recorded. The proteolytic activity was measured viscosimetrically, with a solution of 5 per cent gelatin made up in McIlvaine's buffer (pH 5.3) containing 0.2 M sodium chloride as substrate. 1 unit of proteolytic activity is defined as the amount of enzyme required to reduce the viscosity of the gelatin solution to one-half in 10 minutes. It can be seen from these data that mucolytic and proteolytic activities do not run parallel. In the better preparations of *Ficus* lysozyme, mucolytic activity had been concentrated 3 to 4 times as compared to the crude material, whereas proteolytic activity had decreased by about the same factor. The lytic activity of these *Ficus* samples appears about twice as potent as pure egg white lysozyme.

Lysozyme activity is obviously not confined to the plant species from which papain and ficin are prepared. A test of three samples of freshly collected latex[5] showed the following mucolytic activity (in McIlvaine's buffer of pH 5.3 and 0.2 M sodium chloride):

	units per cc.
Ficus elastica	9.1
" *lyrata*.	17.4
Euphorbia pulcherrima (poinsetta)	18.2

* Obtained through the courtesy of Merck and Company, Inc.

DISCUSSION

The occurrence of lysozyme in the latex of some plants, in some instances in very high concentration, seems remarkable. The apparent activity of crude ficin, for example, is about 65 per cent of that of pure egg white lysozyme per microgram of dry weight, if the activity of the latter is taken as 1 unit in 0.73 γ. On the other hand, if we assume, on the basis of the proteolytic activity, that pure *Ficus* lysozyme contains 1 unit in about 0.3 γ, the apparent activity is only about 20 per cent. The rôle this lysozyme plays in the physiology of the plant is unknown. It remains to be seen whether the mucolytic activity is connected with the metabolism of

[5] We are indebted to Dr. W. J. Robbins of the Bronx Botanical Gardens for this material.

the polysaccharide in the plant, or whether it is directed as a defensive enzyme against insects or other plant parasites.

SUMMARY

1. The presence of a mucolytic enzyme with activities similar to those of egg white lysozyme has been found in the latex of different plants.

2. In crude ficin the concentration of such an enzyme is especially high.

3. The *Ficus* lysozyme has been partly freed of proteolytic enzyme. The proteolytic enzyme, ficin, in the crystalline state is markedly low in lysozyme activity.

4. *Ficus* lysozyme is chemically quite different from the lysozyme found in egg white.

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THE CONJUGATED, NON-PROTEIN, AMINO ACIDS OF PLASMA

I. POSTABSORPTIVE CONCENTRATIONS OF HUMAN PLASMA, SERUM, AND ERYTHROCYTES

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Of the three categories of amino acids present in blood, the free amino acids and the protein amino acids have received much more careful attention than the third class, those which are neither protein nor free. These have been assumed to be manifestations of protein metabolism, representing intermediate stages in the degradation and synthesis of protein. The study of digestive enzymes brought attention early to the formation of a series of compounds of decreasing size from proteins to amino acids. Do living cells contain a complete series of these compounds in the free state, representing the step by step synthesis of proteins? Schoenheimer and his associates (1) have demonstrated so extreme an activity in the regeneration of proteins as to suggest an incorporation of amino acids into proteins without the intermediary formation of many successive oligo- and polypeptides. Thus the extent of polypeptide participation in protein synthesis is brought into question.

Absorption of incompletely digested fragments of protein is probably not an adequate explanation for the presence of peptides in blood, especially if they are present in the postabsorptive state. A major fraction of the blood peptides, namely the cellular glutathione, obviously does not arise from absorption as such from the intestine and probably does not function simply as a transport form of amino acids. Other peptides might be similarly specialized structures.

A practical aspect of peptide metabolism arises from the wide parenteral use of partial hydrolysates of protein. These have been reported to be nutritionally more efficient when administered parenterally than completely hydrolyzed proteins or amino acid mixtures (2). White and Sayers (3) have suggested that peptides perhaps are better handled by the tissues or better retained by the kidneys than are free amino acids. The great biological activity of a number of polypeptides also enhances interest in the metabolism of these substances.

The foregoing considerations, the irregularity in levels of bound amino acids in blood observed heretofore ((4–19), Table I) and the availability of new tools, the manometric ninhydrin procedure (20, 21) and of procedures for certain amino acids permitting differentiation between free and bound

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forms,[1] prompted a reinvestigation of the combined amino acids of human blood, plasma, and serum. The necessity of a precise measurement of α-amino nitrogen of high specificity is emphasized by the questionable increase

TABLE I

Some Observations of Combined Amino Acids of Blood*

Investigators	Material	Deproteinization	Hydrolysis	Analytical method	Normal findings
					mg. per cent N
Schweriner, 1920 (4)	Defibrinated human blood	Mercuric chloride	Acid, 4 hrs.	Formol titration	None detected†
Hiller and Van Slyke (5)	Defibrinated ox blood	Tungstic acid	"	Gasometric nitrous acid	1.4, 4.0, 4.3
	Same samples	2.5% trichloroacetic acid	"	" "	6.5, 7.7, 7.0
Hannaert and Wodon (6)	Dog plasma	Trichloroacetic acid	"	" "	None detected
Blau (7)	Human whole blood	Boiling acetic acid and trichloroacetic acid	"	" "	About 0.9†‡
Swanson (8)	Human blood	Tungstic acid	Alkali	Folin colorimetric	4-20
Jackson, Sherwood, and Moore (9)	" "	2.5% trichloroacetic acid	Acid	" "	Usually less than 1‡
Kotschneff (10)§	Dog blood (femoral vein)	Probably trichloroacetic acid	"	Gasometric nitrous acid	3.8-7
Abderhalden and Rossner (11)	Horse serum .	Dialysis	" or alkali	" "	About ⅓ as much combined as free
			Erepsin or trypsin-kinase	" "	None
Martens (12)	Human plasma	Trichloroacetic acid	Acid	Folin colorimetric	1.2-5.85, average 3.5†
Becher and Herrmann (13)	Human blood	" "	"	" "	Average 2.4
Kirk (14)	" plasma	Tungstic acid	"	Gasometric nitrous acid	−0.54 to +0.55, average 0.03‖

* A long list of investigations (over 70) in which it was sought to determine "polypeptide N," especially in disease, by measuring the difference in nitrogen content (or of substances yielding color with Folin's phenol reagent) (see elsewhere (15-18)) of two blood filtrates obtained by different deproteinizing agents, is not considered here. Highly variable findings have been reported.

† Large increases in disease.

‡ The experimental error was of this order; hence the investigators considered the presence of peptide nitrogen questionable.

§ Kalmykoff (19), also in London's laboratory, found no peptide nitrogen in dialysates of dog serum.

‖ Large negative and positive values reported in uremia.

or lack of increase by hydrolysis reported in several of the above investigations. Evidence has been sought as to the function of the amino acid com-

[1] A differentiation of free and combined amino acids in blood or plasma, and hence estimates of free individual amino acids, are probably not obtained by simple application of microbiological assays (22).

binations by studying the fasting levels, the changes with administration of proteins and protein degradation products, and in disease, and the contribution of certain amino acids to the free and conjugated amino acid levels and to changes in them. This communication reports postabsorptive concentrations of plasma, cells, and serum.

Hiller and Van Slyke (5) demonstrated that a group of protein precipitants ranged from tungstic acid to 2.5 per cent trichloroacetic acid as to efficiency in precipitating intermediate degradation products from solutions of peptone, and they observed that in defibrinated ox blood a portion of the combined amino acids was precipitated by the first but not by the second reagent (Table I). We have determined combined amino acids of the two categories observed by these authors, those present in tungstic acid filtrates and those not present in tungstic acid filtrates but present in 2.5

TABLE II

Effect of Hydrolytic Agent and Hydrolytic Conditions upon α-Amino Nitrogen Found

The values are given in mg. per cent. Each value represents a separate hydrolysis and a single analysis.

| Plasma No. | Before hydrol-ysis | 4 N HCl at 100° in air | | | | 4 N HCl at 100° in N₂ 24 hrs. | 4 N HCl at 110° | | 4 N HCl refluxed 24 hrs. | 2 N and 3 N NaOH at 100° 24 hrs. |
		6 hrs.	12 hrs.	24 hrs.	48 hrs.		12 hrs.	24 hrs.		
1	4.70	4.92	5.25	5.21	5.25		5.23	5.34	5.25	
		4.75	5.18	5.25	5.18		5.20	5.17	5.17	
2	4.30			6.50		6.40				6.13
				6.53		6.44				6.10
				6.44		6.52				6.18

per cent trichloroacetic acid filtrates. For convenience the latter fraction has been designated as "polypeptide" nitrogen, although the substances involved may include even proteins (23).

The term *α-amino nitrogen* is used here for the nitrogen determined by the manometric ninhydrin procedure. The term *combined* (or *bound*) *α-amino nitrogen* is used rather than the term *peptide nitrogen* for two reasons: (1) Each of the α-amino nitrogens in a typical peptide molecule will be recorded by the ninhydrin method as combined, although one of these nitrogens is not involved in a peptide bond; (2) the only evidence herein bearing upon the nature of the amino acid combinations in plasma is the degree of stability of the bound forms to acid. There is very little evidence for supposing that these compounds are not peptides, although Abderhalden and Rossner (11) failed to observe a release by erepsin of combined amino acids in dialysates of horse serum (Table I).

We have found the total α-amino nitrogen after acid hydrolysis of plasma filtrates to be a reproducible quantity whether hydrolysis was in

air or in nitrogen, at 100° or 110°, for any period over 12 hours (Table II). Alkaline hydrolysis also gave reproducible results, although only 85 per cent as much combined α-amino nitrogen with one plasma as was obtained by acid hydrolysis (Table II). Tryptophane is probably lost by acid hydrolysis of blood filtrates. A tryptophane level in plasma of 1.14 mg. per cent (24) would result in an underestimation of the combined α-amino nitrogen of 0.08 mg. per cent. An overestimation of the combined amino acids of the same magnitude results from the loss of glutamine in the estimation of free α-amino nitrogen (25).

The postabsorptive levels of bound α-amino nitrogen of tungstic acid filtrates of plasma of young adults (twenty-one observations) ranged from

TABLE III

Free and Combined Amino Acids in Filtrates of Human Plasma (Mg. Per Cent)

Subject and date	Determination	Tungstic acid filtrate		Trichloroacetic acid filtrate		Ultrafiltrate	
		Free	Total	Free	Total	Free	Total
H. C., Sept. 7	α-Amino N	4.45	5.22				
	Alanine "	0.57	0.69				
" " 20·	α-Amino "			4.22	5.12		
" " 28	" "	4.42	5.12	4.39	5.01	4.54	5.07
E. L., " 25	" "	4.10	5.16		5.27	4.07	4.93
" Jan. 14	" "	4.30	6.47		6.79		
" " 28	" "	4.11	4.76				
T. R.	" "	4.72	6.08	4.63	6.09		
D. J.	" "	4.67	5.18	4.75	5.23		
	Glycine "	0.28	0.33	0.24	0.34		
R. P.	α-Amino "	4.25	6.49		5.73		
	Glycine "	0.29	0.35		0.31		
T. K.	α-Amino "	4.70	5.23				

0.1 to 2.2 mg. per cent and averaged 0.9 mg. per cent (standard deviation 0.44) (Tables III and IV). The concentration of combined amino acids evidently is subject to a much larger degree of variation than is the concentration of free amino acids (21, 28). The bound α-amino nitrogen concentration has proved more responsive to changes in protein metabolism than the free α-amino nitrogen.[2] There was usually little if any difference between the total α-amino nitrogen of trichloroacetic and tungstic acid filtrates (or of ultrafiltrates) of normal plasmas (Tables III and IV), although considerable differences occurred with diseased individuals[2] and in analyses of serum and erythrocytes. The fact that this "polypeptide" fraction of plasma was usually small or absent, but large in experimental or pathological conditions,[2] indicates that the substances involved in each of the

[2] Unpublished results, Christensen and Lynch.

two categories of combined amino acids are mainly different molecular species. On the other hand, with normal erythrocytes the analytical

TABLE IV

Free and Combined Amino Acids of Human Plasma and Serum

The values are given in mg. per cent.

Subject and date	Determination	Tungstic acid filtrate				Trichloroacetic acid filtrate	
		Free		Total		Total	
		Plasma	Serum	Plasma	Serum	Plasma	Serum
H. C., Dec. 20	α-Amino N	4.50	4.40 *	5.20	5.05		7.46
	Glycine "	0.29	0.28	0.34	0.33		0.54
	Alanine "	0.38	0.35	0.48	0.44		
" " 27*	α-Amino "	4.85	4.98	5.27	5.50	5.33	7.01
	Alanine "	0.62	0.63	0.63	0.63		0.83
" Jan. 21	α-Amino "	4.66	5.24	5.73		5.76	
	Glycine "	0.37		0.43		0.42	
" Feb. 8	α-Amino "	4.68	4.93†	5.47		5.58	
	Glycine "	0.32		0.41		0.44	
	Alanine "	0.50		0.69			
" " 15	α-Amino "	5.06	5.18	5.76		5.85	
	" "†		5.32†		6.07†		7.07†
J. M.*	" "	5.2	5.2	6.03			9.00
	" "‡		4.9‡		9.78‡		13.8‡
D. W., Jan. 17	" "	4.40	4.39	5.83	5.80	5.79	
	Glycine "	0.30		0.37		0.38	
" " 25	α-Amino "	4.41	4.52	5.20	5.10		
" " 31	" "	3.93	4.33	4.36		5.27	
	Glycine "	0.31		0.42			
H. B.	α-Amino "	4.92	5.04	5.02		5.84	
	" "†		5.10†				
	Glycine "	0.32		0.39		0.40	
	Alanine "	0.57		0.71		0.67	
D. J.	α-Amino "	4.10	4.38	5.10	5.39	6.29	7.44
	" "	4.11§	4.30§				

* Serum by recalcification of oxalated plasma.
† Fibrin clot left undisturbed 3 hours.
‡ "Chloroform serum," Tagnon (26).
§ Analysis without deproteinization, MacFadyen (27).

differences observed with the two filtrates were due largely to a variation in the extent of the precipitation of glutathione (see below).

Of the non-protein alanine and glycine of plasma, 16 and 18 per cent respectively were as an average in combined form, compared with 17 per cent of the total α-amino nitrogen. This similarity of partition as to free and combined state failed to suggest the presence of specialized structures

among plasma peptides, as contrasted with erythrocyte peptides. The normal fasting levels of these two amino acids showed a constancy similar to that of the α-amino acids (glycine nitrogen, average 0.31 mg. per cent, S.D. = 0.026; alanine nitrogen, average 0.54 mg. per cent, S.D. = 0.08).

Erythrocytes—Analysis of erythrocyte filtrates emphasized the major influence of glutathione on the picture obtained here. Bound glycine was about 40 to 50 times as great as in the plasma. Glutathione should contribute, per molecule, 1 bound glycine molecule and 1 free and 2 bound α-amino nitrogen atoms. Analysis confirmed this assumption, the ratio of total to free α-amino nitrogen found for glutathione (obtained from the Eastman Kodak Company), being 2.95. Assuming that all the bound glycine of the red blood cells represents glutathione, one can calculate "non-glutathione" free and bound α-amino nitrogen. The free α-amino nitrogen values so obtained (Table III) were similar to those of simultaneous plasma samples, while the corrected bound α-amino nitrogen values were slightly in excess of the values for plasma. This represents good evidence that the free amino acids of blood are rather evenly distributed between cells and plasma, and indicates that the peptide nitrogen (other than that due to glutathione) has a similar concentration in the two phases.

Danielson (29) pointed out the large contribution of glutathione to the amino acid nitrogen of erythrocytes and he attempted to escape this interference by preparing "unlaked" tungstic acid filtrates of erythrocytes and blood. Using his modification of the Folin colorimetric method (29), he concluded that erythrocytes of normal young men contained from 0.34 to 2.19 mg. per cent of amino nitrogen with an average of 1.04 mg. per cent. Even lower values were reported for young women. Dunn *et al.* (24) reported an analysis of the tryptophane content of a tungstic acid filtrate of unwashed erythrocytes which was about one-fourth the con-.centration observed in plasma. Hamilton and Van Slyke (21) found 6.5 to 9.6 mg. per cent of α-amino nitrogen in erythrocytes, deproteinizing by picric acid. From these latter data, one sees that even if all the glutathione is recovered in picric acid filtrates, the free amino acids of erythrocytes can scarcely be appreciably lower than those of plasma. Our measurement of bound glycine has permitted an evaluation of the maximum contribution of glutathione to the amino acid values (free and bound) for erythrocyte filtrates. This contribution of glutathione has probably been overestimated previously; glutathione was not only incompletely but rather irregularly recovered in tungstic acid filtrates, thus probably explaining irregularities in amino acid contents found for erythrocyte filtrates by tungstic acid. (See, for example, Folin and Berglund (30).)

Neither tungstic acid nor trichloroacetic acid filtrates revealed as much bound glycine as was to be expected from the values usually found for human erythrocyte glutathione (Table V), whereas a 2 per cent sulfosali-

cylic acid filtrate (31), recommended for the determination of glutathione, showed bound glycine equivalent to 69 mg. per cent of erythrocyte glutathione. It has been recognized that trichloroacetic acid causes precipitation of part of the glutathione, although it was not clear whether losses with tungstic acid were due to precipitation or to autoxidation of glutathione (31). Measurement of the bound erythrocyte glycine in sulfosalicylic acid filtrates presents a simple measurement of total glutathione, unaffected by autoxidation of glutathione or by spontaneous splitting of glutamic acid from glutathione. If one assumes that the non-gluta-

TABLE V

α-Amino Acids, Free and Combined, of Erythrocytes (Mg. Per Cent)

Subject	Determination	Tungstic acid filtrate				Trichloroacetic acid filtrate			
		Free	Bound	Non-glutathione*		Free	Bound	Non-glutathione*	
				Free	Bound			Free	Bound
D. J.	α-Amino N					8.03	5.97	5.75†	1.41†
.	Glycine "					0.30	2.28		
R. P.	α-Amino "	6.79	6.31	4.42‡	1.57‡	7.15	7.52	4.45	2.03
	Glycine "	0.34	2.37				2.75		
	Alanine "	0.57	0.00				0.08		
H. C.	α-Amino "	6.98	5.39	4.75§	0.93§				
	Glycine "	0.37	2.23‖						
	Alanine "	0.3	0.2						

* Calculated under the assumption that the bound glycine is all due to glutathione.

† Simultaneous plasma free amino acid N = 4.85 mg. per cent; bound (trichloroacetic acid filtrate), 0.38 mg. per cent.

‡ Simultaneous plasma free amino acid N = 4.35 mg. per cent; bound (tungstic acid filtrate), 1.14 mg. per cent.

§ Simultaneous plasma free amino acid N = 4.60 mg. per cent; bound, 0.60 mg. per cent.

‖ Analysis of the sulfosalicylic acid filtrate showed 3.10 mg. per cent of bound glycine N in this erythrocyte sample.

thione bound glycine of erythrocytes is the same as that of the plasma, a correction of about 0.06 mg. per cent of glycine nitrogen, or about 2 per cent of the total glutathione, should be deducted. The necessity of this assumption, which appears to be approximately correct, represents the flaw in this method for glutathione.

Changes Produced by Blood Coagulation—MacFadyen (27) observed that serum contained as much as 10 to 40 per cent more α-amino nitrogen than did plasma. We have also observed differences, although in only two of eleven cases were they as high as 10 per cent (Table IV). MacFadyen's analyses were made upon undeproteinized plasma and serum, ours upon

tungstic acid filtrates (in one case upon trichloroacetic acid filtrates also). However, deproteinization did not appear to reduce the difference, as indicated by the analyses upon one blood (D. J., Table IV). Our data suggest that the continued contact with the fibrin clot is involved in the release of α-amino groups. A quantitatively larger change observed by us upon coagulation was the appearance of 1 to 3 mg. per cent of "polypeptide" nitrogen, with little or no change in the combined amino acids of the tungstic acid filtrate. The formation of this "polypeptide" fraction as a product of coagulation indicates that defibrinated blood or serum (Table I) should not be used for estimating combined, non-protein, amino acids of circulating blood. Glycine and alanine appeared to make up about 9 and 17 per cent of the "polypeptide" α-amino nitrogen formed in coagulation.

To compare the proteolytic activity observed in coagulation with the fibrinolytic activity studied by Tagnon (26) "chloroform serum" was prepared according to this author. A still higher concentration of combined amino acids in both filtrates (Table IV) was observed, although no free α-amino acids were released.

<div align="center">EXPERIMENTAL</div>

Blood Samples—The subjects were normal young adults, E. L. and D. J. being young women. The samples were taken in the morning, no food having been taken during the preceding 12 hours. Blood was immediately heparinized[3] after being drawn from a cubital vein, except when the composition of serum and plasma was under comparison. In this case, either of two techniques was used: (*a*) A portion of the mixed blood was heparinized, while another portion was permitted to coagulate in the presence of the cells and the serum separated within 15 minutes, or in the absence of cells, separated by centrifuging in a paraffin-lined tube; or (*b*) 28 ml. of blood were collected in a syringe containing 2 ml. of 3 per cent sodium oxalate; a portion of the plasma obtained was recalcified. The dilution of the plasma was calculated by measuring its volume after centrifugation. In either case the serum was left 3 hours at room temperature before analysis. Except as indicated in Table IV, the fibrin clot was removed when coagulation was complete. Plasma and cells were separated at once by 15 minutes centrifugation at about 3000 R.P.M. After removal of the plasma, both plasma and cells were recentrifuged.

Deproteinization—Tungstic acid filtrates were prepared by the addition to plasma of 4 volumes of water and 5 volumes of freshly mixed sulfuric acid and sodium tungstate (32). Erythrocytes were treated with 16 volumes of the same tungstic acid solution. The determinations of α-amino nitrogen were begun within 2 hours to guard against loss of glutamine

(25). 2.5 per cent trichloroacetic acid filtrates were made according to Hiller and Van Slyke (5) with freshly prepared 5 per cent trichloroacetic acid, adjusted alkalimetrically to strength.

Hamilton and Van Slyke (21) noted that determination of α-amino acids in trichloroacetic acid filtrates presented difficulties because of the formation of carbon dioxide from the deproteinizing agent. We found that, if such a filtrate promptly was acidified to 0.2 N with hydrochloric acid and extracted painstakingly four times with equal volumes of ether, freshly rendered peroxide-free, and then brought to dryness *in vacuo* below room temperature, the α-amino nitrogen was brought to a minimum value quite similar to that found in the tungstic acid filtrate (Table III). The resultant material was hydrolyzed as described above. In most cases we determined only the "total" concentrations of trichloroacetic acid filtrates, analyzing only the hydrolysates. Ultrafiltrates were prepared in the Lavietes ultrafilter (33).

Analytical Methods—α-Amino nitrogen was determined in blood filtrates and in hydrolysates of them by the use of ninhydrin, according to the method of Hamilton and Van Slyke (21), with a citrate buffer of pH 2.5, and a hydrazine-containing (34) sodium hydroxide solution made up in 25 per cent sodium chloride solution, for the primary absorption of carbon dioxide. The conversion factors of MacFadyen (27) were employed in the calculations. Analyses were performed at least in duplicate, and these usually differed by less than 1.5 per cent. To enhance the precision the aliquot portions were kept as large as possible (representing from 0.6 to 1.5 ml. of plasma, cells, or serum). This was managed by analyzing concentrates prepared from the filtrates. The probable error of the estimation of the bound α-amino nitrogen could thus be kept below 0.1 mg. per cent. Interference by urea was avoided as described by Hamilton and Van Slyke (21) by incubating samples with excess ninhydrin. This step was omitted with hydrolyzed samples which contained little urea.

Glycine was determined by measuring the formaldehyde formed by ninhydrin, as described by Alexander, Landwehr, and Seligman (35). Blanks were run under each set of conditions to guard against the presence of significant quantities of preformed formaldehyde. Alanine was determined by measuring the acetaldehyde according to Alexander and Seligman (36) with the modification that the reaction mixture was boiled 30 minutes with aeration of the refluxing vapors before ninhydrin was added to eliminate interference by aldehydes formed without the action of ninhydrin. Certain manipulations introduced interference of this type. Rather than attempt to avoid interference by leucine by adding sulfuric acid in the color development at a higher temperature, as suggested by Alexander and Seligman, we have evaluated this interference by determining the optical densities spectrophotometrically at both 540 and 580 mμ.

At the latter wave-length 6.3 times as much leucine nitrogen as alanine nitrogen were required to produce a given density. The per cent of the optical density which was due to alanine nitrogen was estimated from the ratio of the optical densities at 540 and 580 mμ. From 75 to 90 per cent of the color was due to alanine. With these two methods the duplicate determinations in nearly all cases differed by less than 4 per cent for glycine and 6 per cent for alanine and added glycine and alanine could be recovered.

Hydrolysis—Preliminary experiments indicated that 2 molecules of α-amino nitrogen could be recovered from glycylleucine hydrolyzed in 4 N hydrochloric acid 24 hours at 100° in the presence of tungstic acid and urea, and in tungstic acid filtrates of plasma. Comparisons were made of α-amino nitrogen recoveries after hydrolysis of tungstic acid filtrates of plasma (concentrated *in vacuo* to about the original volume of the plasma) in 4 N hydrochloric acid: (*a*) at 100° in sealed tubes for periods from 6 to 48 hours; (*b*) under the same conditions but with a nitrogen atmosphere; (*c*) at 110° in sealed tubes; (*d*) refluxed 24 hours on a Woods metal bath; and in 2 N and 4 N carbon dioxide-low sodium hydroxide for 24 hours at 100° in sealed Corning alkali-resistant glass tubes. The hydrolysates were adjusted to about pH 2, thymol blue being used as indicator, before analysis.

From the results (Table II) the following conditions of hydrolysis were selected. To a concentrated blood filtrate in a test-tube was added one-half its volume of 12 N hydrochloric acid. The tube was sealed in a flame (merely for convenience), and suspended for 24 hours in a flask of water boiling under a reflux condenser. The hydrolysate was taken to dryness *in vacuo*, water added, and the evaporation repeated. The hydrolysate then was made to volume for the various analyses.

SUMMARY

The plasma of young adults was found to contain from 0.1 to 2.2 mg. per cent (average 0.9 mg. per cent, s.d. = 0.44) of combined "non-protein" α-amino nitrogen, measured by the increase in α-amino nitrogen produced by acid hydrolysis, with in most cases little difference whether filtrates were prepared by tungstic acid or by 2.5 per cent trichloroacetic acid or by ultrafiltration. Alanine and glycine contributed to the bound amino acids in about the same proportion as to the free.

Clotting of plasma produced not only small and variable increases in free α-amino nitrogen but larger increases (1 to 3 mg. per cent) in bound amino acids precipitated by tungstic acid but not by dilute trichloroacetic acid. This latter new fraction contained about 9 and 17 per cent of its α-amino nitrogen as glycine and alanine, respectively.

The evaluation of the contribution of glutathione to the free and bound

amino acids of erythrocyte filtrates, as well as an estimation of glutathione, was attained by determining bound glycine. By this means it was shown that human erythrocytes contain concentrations of free α-amino acids similar to those of plasma, and slightly more of combined amino acids, excluding glutathione.

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THE SPECTROPHOTOMETRIC ESTIMATION OF HEXURO-NATES (EXPRESSED AS GLUCURONIC ACID) IN PLASMA OR SERUM

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Tests with galacturonic acid[1] have shown that this compound reacts quantitatively with the naphthoresorcinol color reagent, much like glucuronic acid. This was to be expected. It may also be anticipated that this reagent will react with other 6-carbon uronic acids which may be available in the body for conjugation from time to time. It is for this reason that the use of the term "hexuronates" (instead of glucuronates) is recommended, whenever doubt exists as to the uronic acid available.

Quantitative estimations of glucuronic acid in the urine of men and animals have been found to be of value in determining the extent of the absorption of certain types of compounds that conjugate in the body with glucuronic acid and that consequently lead to an increased urinary excretion of glucuronates. The determination of these compounds in blood has apparently not been attempted, except by Ratish and Bullowa (1), who removed proteins from whole blood and continued the analysis of the filtrate essentially as outlined for urine by Maughan, Evelyn, and Browne (2).

Principle of Estimation—The analytical procedure given here is a modification of our method previously described for the estimation of glucuronates in urine (3). The method determines glucuronates (or hexuronates expressed as glucuronic acid) in plasma or serum without preliminary removal of proteins or glucose. The analysis is based upon (*a*) preliminary hydrolysis of the acidified sample at 75° (which makes a large amount of free glucuronic acid ready for immediate reaction when the color reagent is added); (*b*) addition of an excess of naphthoresorcinol (sufficient to react with the impurities, proteins, glucose, etc., and with glucuronic acid); (*c*) heating of the plasma-naphthoresorcinol-hydrochloric acid mixture at 50° (in our hands, heating to higher temperatures has not only caused loss of glucuronic acid but also has favored the formation of other compounds that react with naphthoresorcinol); (*d*) extraction of the pigment with ether; and (*e*) determination of the density of the colored ether solution at wavelength 570 mμ.

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[1] The galacturonic acid was kindly supplied by the Research Department of the California Fruit Growers Exchange, Corona Laboratory, Corona, California.

Since naphthoresorcinol is used as the color reagent, the possibility cannot be overlooked that compounds closely related to hexuronates, and reacting similarly, may be reported as glucuronic acid.   However, despite the non-specificity of this reagent, the method lays claim to a considerable degree of accuracy, as judged by the recovery of quantities ranging from 2 to 100 γ of glucuronic acid that were added to samples of plasma.
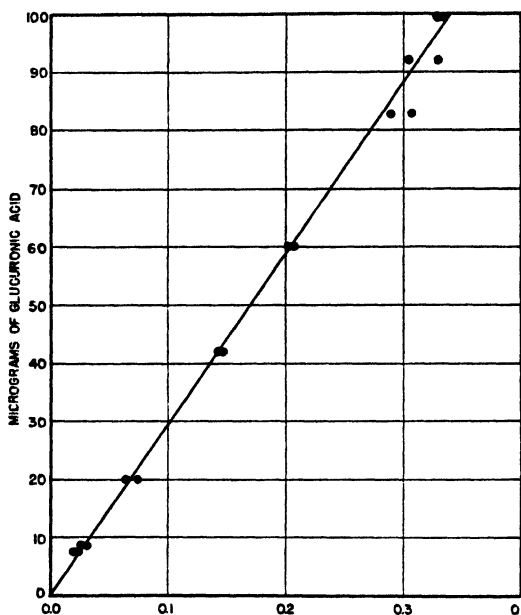
FIG. 1. Relationship between density of color at 570 mμ and concentration of hexuronates, expressed as glucuronic acid.

Reagents Required—
Hydrochloric acid (38 gm. in 100 ml.).
Hydrochloric acid (19 gm. in 100 ml.).
Naphthoresorcinol[2] (10 gm. in 100 ml. of 95 per cent ethyl alcohol).   This reagent is centrifuged or filtered and will remain stable for 5 days if kept cool and protected from light.
Ether, C.P.
Method of Analysis—0.2 ml. to 1 ml. of plasma or serum is diluted with water to 1.8 ml. in a glass-stoppered 50 ml. graduated cylinder, and 0.2 ml. of 19 per cent hydrochloric acid is added.   The whole is kept for 45 minutes

[2] Purchased from B. L. Lemke, 248 West Broadway, New York, New York.

<div align="center">TABLE I</div>

Recovery of Glucuronic Acid Added to Rabbit Plasma

Glucuronic acid in volume of plasma analyzed	Borneol glucuronate added equivalent to following quantities of glucuronic acid	Total glucuronic acid in sample analyzed	Total glucuronic acid recovered	Volume of plasma analyzed and range over which method is expected to give satisfactory recovery
Plasma containing about 150 mg.% glucose				

γ	γ	γ	γ	
5.7	2.0	7.7	7.3	1.0 ml. samples plasma, containing about
9.0	2.0	11.0	10.8	150 mg. % glucose and from 0 to 40 γ
0	4.6	4.6	5.0	glucuronic acid
0	4.6	4.6	4.2	
0	7.0	7.0	7.0	
0	7.0	7.0	8.0	
7.5	8.4	15.9	14.8	
0	40.0	40.0	39.0	
4.0	45.0	49.0	44.0	
4.0	45.0	49.0	40.0	
3.0	71.0	74.0	70.0	
7.5	83.3	90.8	68.3	
3.0	134.0	137.0	126.0	
5.0	177.0	182.0	157.0	
1.0	2.0	3.0	4.0	0.5 ml. samples plasma, containing about
1.0	2.0	3.0	2.7	150 mg.% glucose and from 0 to 50 γ
2.5	4.6	7.1	8.7	glucuronic acid
2.8	9.2	12.0	12.0	
3.5	32.0	35.5	37.5	
0.7	46.4	47.1	46.4	
3.5	46.4	49.9	47.0	
2.5	65.0	67.5	56.3	
2.7	92.8	95.5	76.5	
2.7	92.8	95.5	72.0	
0	2.0	2.0	2.6	0.2 ml. samples plasma, containing about
0	2.0	2.0	1.5	150 mg.% glucose and from 0 to 100 γ
2.0	2.0	4.0	4.5	glucuronic acid
2.0	2.0	4.0	6.0	
1.7	4.6	6.3	6.5	
0.5	9.2	9.7	9.2	
5.2	31.0	36.2	35.0	
1.0	62.0	63.0	63.5	
0.2	62.0	62.2	55.0	
2.5	92.8	95.3	91.3	
3.7	92.8	96.5	92.5	
1.6	87.5	*89.1	90.0	
0	105.0	105.0	108.0	

TABLE I—*Concluded*

Glucuronic acid in volume of plasma analyzed	Borneol glucuronate added equivalent to following quantities of glucuronic acid	Total glucuronic acid in sample analyzed	Total glucuronic acid recovered	Volume of plasma analyzed and range over which method is expected to give satisfactory recovery
			Plasma containing about 250 mg.% glucose	
γ	γ	γ	γ	
1.0	4.6	5.6	6.0	0.5 ml. samples plasma, containing about
2.7	4.6	7.3	8.0	250 mg.% glucose and from 0 to 25 γ
2.3	21.8	24.1	24.6	glucuronic acid
3.0	21.8	24.8	26.5	
4.2	21.8	26.0	21.3	
2.3	40.5	42.8	34.5	
2.5	40.5	43.0	30.3	
1.4	4.4	5.8	5.0	0.2 ml. samples plasma, containing about
1.4	4.6	6.0	5.5	250 mg.% glucose and from 0 to 50 γ
2.2	41.0	43.2	43.0	glucuronic acid
2.2	48.0	50.2	49.0	
1.4	92.8	94.2	86.0	
2.2	92.8	95.0	83.0	

in a water bath at 75°; 2.0 ml. of concentrated hydrochloric acid and 1.0 ml. of 10 per cent naphthoresorcinol are then added, and the cylinder, as well as a similarly prepared blank (which contains the reagents, but water instead of the plasma), are allowed to stand for 90 minutes in a water bath maintained at 50°. After cooling under the tap, each of the solutions is extracted in the cylinder with about 8 ml. of ether by shaking vigorously for about 5 seconds. After separation in two layers, an additional 5 ml. of ether are added to each cylinder and the upper layer is mixed by gentle rotation. After a third portion of the solvent has been added to each cylinder, sufficient to bring the total volume of ether to exactly 15 ml., the total ether extract is again mixed in a similar manner. Addition of the second and third portions of ether and letting the solutions stand for about 3 minutes after each addition of ether will completely clear up any turbidity caused by water in the upper layer.

Colorimetric Reading—The colored ether is now pipetted into a glass-stoppered flask (dried immediately before use by alcohol and ether) and is ready for spectrophotometric analysis, which is carried out by using 10 mm. matched cells, with the instrument set at the wave-length 570 mμ. The experimental ether extract is compared with that obtained from the blank.

Fig. 1 gives the relationship between density of color and concentration of glucuronic acid (or hexuronates expressed as glucuronic acid) in the

sample of plasma or serum analyzed. The final results are expressed in mg. per 100 ml. of plasma or serum, respectively. (Fig. 1 was prepared by plotting the readings obtained when known aqueous solutions of pure

TABLE II

Distribution of Hexuronates (Expressed As Glucuronic Acid) in Cells, Plasma, or Serum of Normal (Control) Human Subjects, Rabbits, and Rats

	Red blood cells	Plasma	Serum
	mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*
H. S.	0.9	1.2	1.3
B. R.	0	0.6	0.6
C. K.	0	0.6	0.6
M. D.	0	0.5	0.6
S. W.	0	0.6	0.5
H. S.	0	0.4	0.4
W. D.	0.6	0.9	0.9
R. C.			0.8
J. S.	1.0		0.7
C. L.	1.5		0.8
S. W.	2.0	1.4	1.4
Rabbit 1	0.4		1.5
" 2	0	1.0	1.5
" 3	1.6	2.2	2.2
" 4	1.6	2.6	2.5
" 5	0.8	0.8	0.8
" 6	1.3	2.5	
" 7	1.1	1.9	
" 8	0.8	1.0	
" 9	0	0.6	
" 10	0.4	0.9	
" 11	0	0.5	
" 12	0.8	1.8	
Rat 1	0	0.7	
" 2	0.7	0.8	
" 3	0	0.7	
" 4	0	0.8	
" 5	0.5	1.1	
" 6	1.0	1.0	
" 7	0.5	1.1	

glucuronic acid or of borneol or menthyl glucuronate[3] were analyzed by this procedure.)

[3] The mentholglucuronic acid was prepared and kindly supplied by Dr. Armand J. Quick of Marquette University School of Medicine; the borneolglucuronic acid ester was purchased from the Fordomes Trading Company, Arlington, New Jersey; and the glucuronic acid was kindly supplied for these observations by J. R. Harrower of the University of Wisconsin.

Certain Practical Considerations—In observations not reported here, samples of 1.0 ml. of plasma or serum have given satisfactory results when routine specimens were checked for possible increases in glucuronic acid content. A volume of 0.2 ml. was generally adopted for analysis after

TABLE III

Distribution of Hexuronates (Expressed As Glucuronic Acid) in Cells, Plasma, or Serum of Rabbits and Rats after One Oral Administration of Cyclohexanone

		Red blood cells	Plasma	Serum
		mg. per 100 ml.	*mg. per 100 ml.*	*mg. per 100 ml.*
Rabbit*	1	2.0	8.5	8.2
"	2	2.8	31.1	28.3
"	3	0.9	13.9	13.9
"	4	0.9	18.9	20.0
"	5	0	20.0	20.2
"	6	0	7.5	8.1
"	7	1.0	19.3	19.8
"	8	1.7	17.3	16.7
"	9	1.3	5.7	
"	10	0.7	12.0	
"	11	0.2	5.3	
"	12	0.3	6.9	
"	13	0	8.0	
"	14	0.6	10.8	
Rat*	1	0	3.0	
"	2	0	2.1	
"	3	0.5	2.4	
"	4	1.2	7.5	
"	5	1.0	4.4	
"	6	1.0	4.4	
"	7	0		7.7
"	8	0.9		7.8
"	9	0		6.4
"	10	0.8		6.6
"	11	0		7.0
"	12	0.4	8.2	
"	13	0.7	7.9	

* Each rabbit was given 2 ml. and each rat 0.4 ml. of cyclohexanone per kilo of body weight. Blood samples were drawn about 2½ hours after medication.

compounds known to induce an increase of glucuronic acid in the blood had been administered to experimental animals.

Running a blank and comparison of the experimental ether extract with that obtained from a blank will tend to eliminate errors introduced by variations in the purity of naphthoresorcinol. This modification was

deemed essential because of the much smaller quantities of glucuronic acid encountered in specimens of plasma than in those of urine.

The ether extract (after removal from the hydrochloric acid solution) is stable for about 1 hour if protected from direct sunlight.

Hemolysis, if present, will interfere and give false high values. A constituent of the red blood cell reacts with the naphthoresorcinol-hydrochloric acid mixture and forms a brown ether-soluble pigment.

3 days standing at 5° or at 25° did not induce loss of hexuronates in samples of plasma obtained from rabbits given cyclohexanol.

TABLE IV

Hexuronates (Expressed As Glucuronic Acid) in Plasma and Urine of Rabbits after One Oral Dose of Cyclohexanone or 2-Methyl-2,4-pentanediol

Hrs. after oral administration of compound	Rabbit D 3363,* dose 0.8 ml. per kilo cyclohexanone		Rabbit D 3904,* dose 1.0 ml. per kilo pentanediol		Rabbit D 3364,* dose 1.6 ml. per kilo cyclohexanone		Rabbit D 3365,† dose 3.0 ml. per kilo cyclohexanone	
	Plasma	Urine	Plasma	Urine	Plasma	Urine	Plasma	Urine
	mg. per 100 ml.	*mg. per total volume*	*mg. per 100 ml.*	*mg. per total volume*	*mg. per 100 ml.*	*mg. per total volume*	*mg. per 100 ml.*	*mg. per total volume*
1	7	150	9	17	20	75	22	240
2	15	290	14	75	39	127	33	330
3	11	80	15	148	58	265	42	500
4	5	65	34	267	67	365	43	550
5	5	60	26		84	345	43	660
6	4	40	27		98	300	31	340
7	3	45	19		120	400	15	410
8			20					
12	2	20			91			
14	1	10						
24	1	20	1	340	5	1740	3	290
Total excreted .		780		847		3617		3320

* Survived.

† Died 26 hours after oral administration.

EXPERIMENTAL

Table I summarizes the recovery of glucuronic acid added to plasma. (The concentration of glucose in these samples of plasma was increased to a level of approximately 150 or 250 mg. per cent, respectively.) The latitudes over which the method is expected to give dependable results are indicated. Over these specific ranges the recoveries of glucuronic acid have been satisfactory in the presence of proteins and elevated concentrations of glucose. Table II gives the distribution of hexuronates (expressed as glucuronic acid) in red blood cells and in plasma or serum of blood

obtained from healthy (untreated) experimental subjects, rabbits, and rats. (The cells were washed three times with 0.9 per cent aqueous sodium chloride, subsequently hemolyzed with water, and the proteins precipitated with 10 per cent aqueous sodium tungstate and $\frac{2}{3}$ N sulfuric acid. The filtrate analyzed was equivalent to about 0.2 ml. of cells.) The figures show that, with a few isolated exceptions, hexuronic acids are normally present primarily in the fluid part of blood. Table III summarizes similar data after oral administration of cyclohexanone. Interestingly enough, increases in glucuronic acid values were found only in plasma or serum. Table IV summarizes data on the concentration of hexuronates (expressed as glucuronic acid) in the plasma of rabbits and the quantities excreted in the urine of these animals after administration of cyclohexanone and 2-methyl-2,4-pentanediol. The figures indicate that estimations of glucuronic acid in either plasma or urine lend themselves for study of the rate of absorption or the metabolism of a compound that conjugates in the body with a uronic acid.

SUMMARY

1. A photoelectric procedure is presented for the estimation of hexuronates expressed as glucuronic acid in plasma or serum without preliminary. removal of ordinarily interfering compounds (proteins, glucose, etc.). The principle employed is similar to that utilized for the estimation of glucuronic acid in urine; which was reported earlier.

2. The greater portion of the hexuronates normally found in blood is present in the plasma or serum. Values found for untreated experimental subjects, rabbits, and rats, in terms of glucuronic acid, ranged from 0.4 to 2.5 mg. per 100 ml. of plasma.

3. Analysis of plasma or serum is recommended for the study of the rate of absorption or the metabolism of a compound that conjugates in the body with a hexuronic acid. The administration of cyclohexanone or 2-methyl-2,4-pentanediol to rabbits or rats induced no change in glucuronic acid values in the cellular fraction of blood, while increases of hexuronates in plasma were significant (up to 120 mg. of glucuronic acid per 100 ml.) and in direct relation to the quantities of hexuronates excreted in the urine.

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3. Deichmann, W., *J. Lab. and Clin. Med.*, **28**, 770 (1943).

THE THIOUREA ANALOGUE OF DESTHIOBIOTIN

By GEORGE BOSWORTH BROWN and VINCENT du VIGNEAUD

(*From the Department of Biochemistry, Cornell University Medical College, New York City*)

Desthiobiotin (I), in which the tetrahydrothiophene

$$
\begin{array}{c}
\text{O} \\
\parallel \\
\text{C} \\
\diagup \quad \diagdown \\
\text{HN} \qquad \text{NH} \\
| \qquad\quad | \\
\text{CH}_3\!\!-\!\!\text{CH}\!\!-\!\!-\!\!\text{CH}\!\!-\!\!(\text{CH}_2)_5\!\!-\!\!\text{COOH}
\end{array}
$$

(I)
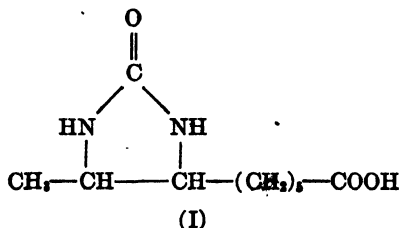
ring of biotin has been cleaved by removal of the sulfur but in which the imidazolidone ring remains unaltered, possesses approximately the same yeast growth-promoting activity as biotin (1). It also shows a strong antibiotin activity with *Lactobacillus casei* (2, 3). Various compounds have been prepared in which the carbon skeleton of desthiobiotin is altered but in which the imidazolidone ring is not changed. Certain of these compounds are definite antibiotins (4, 5).

As a step toward ascertaining the effect of alteration of the imidazolidone portion of the molecule while retaining the carbon skeleton of desthiobiotin, we have prepared the thiourea analogue of desthiobiotin (II).

$$
\begin{array}{c}
\text{S} \\
\parallel \\
\text{C} \\
\diagup \quad \diagdown \\
\text{HN} \qquad \text{NH} \\
| \qquad\quad | \\
\text{CH}_3\!\!-\!\!\text{CH}\!\!-\!\!-\!\!\text{CH}\!\!-\!\!(\text{CH}_2)_5\!\!-\!\!\text{COOH}
\end{array}
$$

(II)
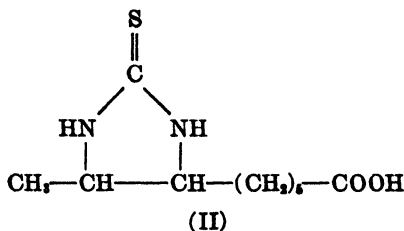
This analogue, 5-methyl-2-thiono-4-imidazolidinecaproic acid, was prepared from ethyl ζ, η-diaminopelargonate by the action of carbon disulfide followed by heating to form the thiourea. The synthetic product was a mixture of the diastereoisomers. The separation of one of the optically inactive diastereoisomers from the initial mixture was accomplished by the fractionation of the morpholine salts. Smaller amounts of this same isomer were obtained by repeated recrystallization of the free acids from water.

Assay of samples of the mixed isomers showed a stimulation of the growth of *Saccharomyces cerevisiae* (6, 7), which is equivalent to an activity of 0.1 to 0.2 per cent of that of biotin or of desthiobiotin. The purified isomer had an activity of 0.06 per cent that of desthiobiotin. This growth-promoting activity was inhibited by amounts of avidin equivalent to the thiourea analogue, showing that the avidin-combining power of the molecule has not been destroyed by the substitution of the thiourea for the urea moiety.

The thiourea analogue of desthiobiotin was also tested for inhibition of the growth of *Lactobacillus casei* by the procedure described (8). It was found to inhibit this organism, and this inhibition could be prevented by the addition of biotin. The molar inhibition ratio (4) was determined with an incubation period of 40 hours. For the mixed isomers of the thiourea analogue of desthiobiotin the molar inhibition ratio was found to be approximately 600,000 and for the purified isomer 450,000. Since the ratio for desthiobiotin with *Lactobacillus casei* is 9100, the values represent 1.5 and 2.0 per cent of the antibiotin activity of desthiobiotin for this organism.

It is conceivable that a trace of desthiobiotin, resulting from the hydrolytic removal of the sulfur from the thiourea grouping, could partially account for the activity. The fact that the isomer obtained by the repeated recrystallizations from hot water possessed approximately the same activity as the initial mixture of isomers indicates at the least that such a hydrolysis does not proceed readily. In addition, the biotin and antibiotin activities of the thiourea analogue do not represent the same proportions of the corresponding activities of desthiobiotin, and it is more than probable that the antibiotin activity is an intrinsic activity of the molecule.

In order to show that the samples of the synthetic diaminopelargonic acid from which the thiourea analogue of desthiobiotin was prepared contained appreciable amounts of the isomer corresponding to the natural desthiobiotin and biotin, the synthesis of desthiobiotin was carried out. With conditions similar to those used for the resynthesis of natural desthiobiotin from natural diaminopelargonic acid (9), an analytically pure sample of the mixed isomers was obtained in 50 per cent yield. The product possessed a growth-promoting activity for *Saccharomyces cerevisiae* of 25 per cent that of biotin. It was similar to the mixture prepared by the method of Wood and du Vigneaud (10) and is undoubtedly a mixture of *dl*-desthiobiotin (11, 12) and *dl*-desthioallobiotin (12).

EXPERIMENTAL

Ethyl η -Oxopelargonate—The alkylation of 104 gm. of ethyl acetoacetate with 176 gm. of ethyl ε-bromocaproate (13) was carried out as described (14). The product was saponified in cold 5 per cent aqueous NaOH rather

than in diethylene glycol solution, as previously described. The crude ester, after distillation of the ethanol, was added to 1400 cc. of cold 5 per cent NaOH and the mixture was stirred vigorously. After 15 hours the alkali-insoluble material was separated. The solution was acidified with 45 cc. of concentrated H_2SO_4, stirred for 2 hours, and heated on the steam cone for an additional 2 hours. The solution was extracted three times with ether and the extracts were concentrated.

The 180 gm. of crude η-oxopelargonic acid were esterified by refluxing for 4 hours with 600 cc. of ethanol and 30 cc. of concentrated H_2SO_4. The solution was concentrated, washed with water, and distilled, yielding 108 gm., boiling at 113–115° at 2 mm., and representing 68 per cent of the theoretical yield.

The 2,4-dinitrophenylhydrazone was prepared. It was recrystallized from 90 per cent ethanol and melted at 94–95°.[1]

$C_{17}H_{24}O_6N_4$ (380.4).   Calculated, N 14.74; found, 14.73

Ethyl ζ,η-Diaminopelargonate—20 gm. portions of the keto ester were converted to the dioximino ester by the procedure described (14). After two recrystallizations from methanol-water, the crude mixture of isomeric dioximes was obtained in 50 to 75 per cent yield. These samples melted in the range of 90–105° and several more recrystallizations, entailing losses of 50 per cent or more, were required to obtain the material melting at 107–108°.

The reduction (14) to the ethyl diaminopelargonate was carried out on 107–108° samples and on lower melting samples of the dioximino ester. Samples of the crystalline ethyl ζ,η-diaminopelargonate sulfate prepared from either reduction product were identical. The reduction of the lower melting mixture of isomeric dioximes is markedly advantageous from the standpoint of over-all yield.

Preparation of Thiourea Analogue of Desthiobiotin (5-Methyl-2-thiono-4-imidazolidinecaproic Acid—The 7 gm. of ethyl ζ,η-diaminopelargonate obtained by the reduction of 8 gm. of the dioximino ester were dried over phosphorus pentoxide to remove the last traces of ammonia. It was then dissolved in 25 cc. of methanol, and 25 cc. of carbon disulfide were added. The first few drops caused some evolution of heat and the formation of a slight precipitate. The solution was warmed to 45–50° and the excess carbon disulfide distilled. The evolution of H_2S began and continued slowly for 8 hours. At that time 50 cc. of 50 per cent methanol were added and the heating was continued to expel the H_2S completely. The solution was cooled, 10 cc. of 5 N NaOH were added, and the saponification of the

ester was allowed to proceed at room temperature overnight. The solution was filtered after the addition of Darco. It was acidified, heated to boiling with Darco, and again filtered. The product which crystallized upon cooling at 5° was recrystallized from 200 cc. of 15 per cent methanol. It amounted to 4.0 gm., representing 53 per cent of the theoretical yield. This mixture of isomers melted at 115–128°. After a second recrystallization, a sample was obtained which melted at 122–128° and possessed the following composition.

$C_{10}H_{18}O_2N_2S$.   Calculated.   N 12.16, S 13.92
230.2   Found.   " 12.33, " 13.80

A product of the same melting point was obtained from ethyl diamino-pelargonate, which had been regenerated from the crystalline sulfate.

The mixed diastereoisomers of the thiourea analogue of desthiobiotin were fractionated by a series of eight to ten recrystallizations from 30 to 40 volumes of hot water. About 10 per cent of the sample was obtained as a product, melting sharply at 147–148°.

$C_{10}H_{18}O_2N_2S$.   Calculated.   N 12.16, S 13.92
230.2   Found.   " 12.14, " 14.10, neutral equivalent 232

The mother liquors from these recrystallizations yielded fractions melting from 120–140°.

Fractionation of Morpholine Salts of Thiourea Analogue of Desthiobiotin —To 1.27 gm. of the product, m.p. 115–128°, in 15 cc. of methanol was added 0.5 gm. of morpholine. After the addition of 50 cc. of ether the salt crystallized in quantitative yield. It melted at 125–131°, and, without recrystallization, it possessed the following composition.

$C_{14}H_{27}O_3N_3S$ (317.3).   Calculated, N 13.24; found, 12.82

To 1.3 gm. of this product in 100 cc. of hot ethyl acetate were slowly added 12 cc. of ethanol, until complete solution was obtained. Upon cooling the solution, 0.87 gm. of crystalline salt, m.p. 110–116°, was obtained. After two more such recrystallizations from 50 cc. portions of ethanol-ethylacetate (1:4), followed by one from 10 cc. of alcohol, 0.60 gm. of salt melting at 126–128° was obtained. Admixture with the unrecrystallized salt lowered the melting point.

$C_{14}H_{27}O_3N_3S$.   Calculated.   N 13.24, S 10.10
317.3   Found.   " 13.38, " 10.34

When 400 mg. of this salt were dissolved in 6 cc. of water and HCl was added, the free acid crystallized. It melted at 144–147° and, after recrystallization from 7 cc. of hot water, 200 mg., melting at 147–148°, were obtained.

Admixture with the sample obtained through the fractional crystallization from water caused no depression of the melting point.

` The residues from the mother liquors of the recrystallizations of the morpholine salt possessed melting points between 95–117° and no pure second isomer was obtained.

Desthiobiotin—To 130 cc. of 10 per cent Na_2CO_3 was added 1 gm. of ethyl ζ,η-diaminopelargonate sulfate and the solution was heated to 90° for a few minutes to saponify the ester. The solution was then maintained at 25° and was treated with phosgene until it became acid to Congo red. The solution was then concentrated to dryness *in vacuo*. The residue was taken up in 75 cc. of water and a small amount of material, insoluble in water and in ether, was discarded.· The aqueous solution was extracted continuously with ether for 16 hours and the ether was then evaporated, leaving 550 mg. of crystalline residue. The latter was dissolved in a small amount of methanol and water. After the solution was treated with Darco and filtered, it was concentrated to dryness. The residue was recrystallized from 10 cc. of water. The first crop, crystallizing rapidly at room temperature, amounted to 50 mg. and melted at 146–155°. It possessed a low biological activity and was not further investigated. The main fraction crystallized upon cooling at 5°. It amounted to 340 mg., representing 50 per cent of the theoretical yield. Assay with *Saccharomyces cerevisiae* showed a growth-promoting activity which was 25 per cent that of biotin. It melted at 132–134°, which corresponds to the melting point of the product of similar activity reported by Wood and du Vigneaud (10). There was a trace of material present which melted at approximately 142°. The product possessed the following composition.

$C_{10}H_{18}O_2N_2$ (214.3). Calculated, N 13.08; found, 12.87

An additional 80 mg. were obtained upon concentration of the mother liquors.

SUMMARY

To investigate the effect of an alteration of the imidazolidone moiety of desthiobiotin, the synthesis of the thiourea analogue has been carried out. In contrast to desthiobiotin this thiourea analogue possesses very slight growth-promoting activity toward yeast. This activity is completely inhibited by avidin. Toward *Lactobacillus casei*, the thiourea analogue evidences a low antibiotin activity in comparison with that of desthiobiotin.

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13. Brown, G. B., and Partridge, C. W. H., *J. Am. Chem. Soc.*, **66**, 839 (1944).
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LETTERS TO THE EDITORS

STREPTOCOCCAL FIBRINOLYSIN

Sirs:

It is well known that an exotoxin from the broth cultures of certain strains of β-hemolytic streptococci produces solution of a fibrin clot in plasma.[1,2] The chemistry of its isolation is not complete and many properties must still be determined. A convenient, simple method is presented for the concentration of streptococcal fibrinolysin as also is an assay technique that has been found quite suitable.

Culture of β-hemolytic streptococci, American Type Culture Collection strain 6011, was made on blood agar slants. A broth inoculum was made by transfer from the blood agar slant to 10 ml. of the broth recommended by Christensen.[3] This was incubated at 37° for 24 hours. Inoculation was made at the rate of 1 ml. of seed per 500 ml. quantities of broth.

After 24 hours incubation at 37°, 8 liters of broth were sterilized by filtration through a Mandler filter, 10 gm. of norit SG-11 per liter were added, and adjustment to pH 5.5 made with N HCl.

The norit was collected by centrifugation in the angle head International centrifuge at 5000 R.P.M. for 10 minutes. The streptococcal fibrinolysin was eluted three times by suspending the norit in 300 ml. of 0.1 M phosphate buffer, pH 7.3.[2] The eluate was separated from the norit by centrifugation as above. The combined eluate, 900 ml., was filtered through a Seitz filter with a Fisher EK pad. The filtrate was dialyzed for 3 hours in Visking No Jax 29/32 casings against frequent changes of cold distilled water. Dialysis was considered complete when no test for phosphate could be obtained in the formerly buffered eluate. The solution of streptococcal fibrinolysin was concentrated to 200 to 250 ml. in a vacuum still at 32–35° and then lyophilized. Final drying of the solids was accomplished in a vacuum desiccator over phosphorus pentoxide.

These streptococcal fibrinolysin preparations were a very light tan powder, non-hygroscopic, and stable for at least 1 year in this state. The solubility curves showed two definite components with a possible third compound of very limited solubility. Streptococcal fibrinolysin is soluble

[1] Tillett, W. S., and Garner, R. L., *J. Exp. Med.*, **58**, 485 (1933). Tillett, W. S., *Bact. Rev.*, **2**, 161 (1938). Christensen, L. R., *J. Gen. Physiol.*, **28**, 363 (1945).

[2] Garner, R. L., and Tillett, W. S., *J. Exp. Med.*, **60**, 239 (1934).

[3] Christensen, L. R., *J. Infect. Dis.*, **66**, 278 (1940).

in ammonium sulfate solutions up to at least 65 per cent concentration of this salt. It is soluble at pH 5.5.

Assay of Streptococcal Fibrinolysin 307

For this experiment 8 liters of sterile broth culture were divided equally and worked simultaneously.

Material assayed	Norit SG-11	Al(OH)₃ B
	Lysis time	
Broth	8 hrs.	8 hrs.
Supernatant broth after adsorption	No lysis in 24 hrs.	No lysis in 24 hrs.
PO₄ eluate .	4 hrs.	> 16 hrs.
Dialysate	230 min.	> 16 "
Lyophilized (1 mg. sample)	84 "	142 min.

Assay was accomplished by adding 0.2 ml. of a solution of streptococcal fibrinolysin to 0.1 ml. of 100 unit thrombin and adding 0.3 ml. of plasma containing 300 mg. per cent of fibrinogen. The table shows the assays of norit-prepared material as well as assays of streptococcal fibrinolysin adsorbed on Willstätter's polyaluminum hydroxide B.[2,4]

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[4] Willstätter, R., *Ber. chem. Ges.*, **57**, 1082 (1924).

A HEAT-LABILE STIMULATORY FACTOR FOR STREPTOCOCCUS FAECALIS R

Sirs:

We have previously described[1] a new, labile factor present in liver and milk which is necessary for the maintenance of normal weight and blood in the monkey. Since routine assays for this factor with the monkey are long and tedious, attempts were made to determine whether similar substances might stimulate growth of bacteria. Preliminary trials with a number of organisms revealed the presence of a heat-labile substance in raw liver and raw milk which stimulated growth of *Streptococcus faecalis* R on a "complete" purified medium. This medium contained, per tube, hydrolyzed casein 50 mg., *l*-cystine 2 mg., glucose 200 mg., sodium acetate 200 mg., tryptophane 500 γ, adenine 100 γ, guanine 100 γ, uracil 100 γ, pyridoxine 20 γ, niacin 5 γ, riboflavin 2 γ, calcium pantothenate 1 γ, thiamine 1 γ, *p*-aminobenzoic acid 1 γ, folic acid 0.05 γ, biotin 0.004 γ, Salts A[2] 0.05 cc., Salts B[3] 0.05 cc., and a tryptic casein digest (to supply strepogenin[4]) 6 mg.

The basal medium and appropriate amounts of distilled water were added to test-tubes which were then autoclaved at 15 pounds pressure for 15 minutes. The liver and whey preparations were filtered through a sterile Seitz filter and added aseptically to the tubes to make a total volume of 10 cc.

Cells from an 8 to 12 hour-old culture of *S. faecalis* R in 10 cc. of an enriched basal medium were centrifuged and resuspended in 100 cc. of sterile saline. 1 drop of this suspension was added to each tube. The tubes were incubated for 12 hours at 37° and comparative turbidities determined with an Evelyn colorimeter and Filter 660 with the test-tube holder in the colorimeter set for 6 cc.

Addition of very dilute preparations of raw liver or raw whey produced significantly more growth than was obtained in the tubes containing only the basal medium (see the table). When the liver or whey supplement was autoclaved for 15 minutes at 15 pounds pressure, very little or no stimulation of growth occurred, indicating the heat lability of this factor.

[1] Cooperman, J. M., Waisman, H. A., McCall, K. B., and Elvehjem, C. A., *J. Nutr.*, **30**, 45 (1945). Cooperman, J. M., McCall, K. B., and Elvehjem, C. A., *Science*, **102**, 645 (1945). Cooperman, J. M., Ruegamer, W. R., and Elvehjem, C. A., *Proc. Soc. Exp. Biol. and Med.*, in press.

[2] Salts A contains KH_2PO_4 5 gm., K_2HPO_4 5 gm., and H_2O 50 cc.

[3] Salts B contains $MgSO_4 \cdot 7H_2O$ 10 gm., NaCl 0.5 gm., $FeSO_4 \cdot 7H_2O$ 0.5 gm., $MnSO_4 \cdot H_2O$ 0.5 gm., H_2O 250 cc.

[4] Sprince, H., and Woolley, D. W., *J. Exp. Med.*, **80**, 213 (1944).

Maximum growth was obtained when a filtered water extract of 5 mg. of raw liver or 1 cc. of filtered raw whey was added to each tube.

The addition of glutamine, ascorbic acid, thioglycolic acid, or pyridoxamine to tubes containing either the basal medium or the basal medium plus autoclaved liver failed to give any stimulatory effect.

Supplement to 10 cc basal medium	Galvanometer readings	
	Filtered (unheated) samples	Samples autoclaved 15 min
None	70	70
Extract from 1 mg. raw liver	59	70
" " 2 " " "	47	70
" " 5 " " "	38	66
Raw whey, 0.02 cc	56	70
" " 0 10 "	50	68
" " 1 0 "	38	62
Glutamine, 5–500 γ	70	
Ascorbic acid, 5–500 γ	70	
Pyridoxamine, 5–500 γ	70	
Thioglycolic acid, 5–500 γ	70	

The bacterial factor and the monkey factor show similar lability and distribution in liver and milk products. Much further work will be required to determine whether the two substances are identical.

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THE DIFFERENTIAL EFFECT OF BIOTIN SULFONE AND γ-(3,4-UREYLENECYCLOHEXYL)BUTYRIC ACID UPON THE MICROBIOLOGICAL ACTIVITY OF BIOTIN AND OXYBIOTIN*

Sirs:

The antibiotin activities of biotin sulfone[1] and γ-(3,4-ureylenecyclo-hexyl)butyric acid[2] have been reported. The present experiments demonstrate that for *Lactobacillus arabinosus*[3] the inhibitory action of these compounds toward *dl*-oxybiotin[4] is much more pronounced than toward *d*-biotin.
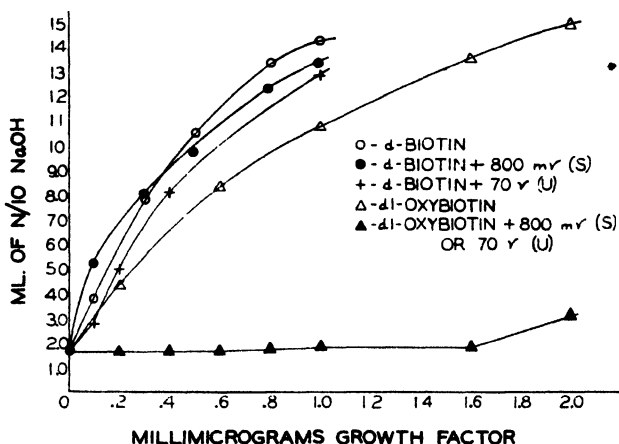
FIG. 1. The differential effect of biotin sulfone (S) and γ-(3,4-ureylenecyclohexyl)-butyric acid (U) upon the microbiological activity of biotin and oxybiotin.

Neither the sulfone nor the ureylene compound had any growth-promoting activity.

The effects of biotin sulfone (200 to 1200 millimicrograms per tube) upon growth resulting from varying levels of *d*-biotin (0.1 to 1.0 millimicro-

[1] Dittmer, K., and du Vigneaud, V., *Science*, **100**, 129 (1944).

[2] English, J. P., Clapp, R. C., Cole, Q. P., Halverstadt, I. F., Lampen, J. O., and Roblin, R. O., Jr., *J. Am. Chem. Soc.*, **67**, 295 (1945).

[3] Wright, L. D., and Skeggs, H. R., *Proc. Soc. Exp. Biol. and Med.*, **56**, 95 (1944). The basal medium was modified by the addition of 0.5 gm. of sodium citrate and 0.1 gm. of *l*-asparagine per liter.

[4] Pilgrim, F. J., Axelrod, A. E., Winnick, T., and Hofmann, K., *Science*, **102**, 35 (1945).

gram per tube) or *dl*-oxybiotin (0.2 to 2.0 millimicrograms per tube) were first determined with an incubation period of 72 hours. Under these conditions, biotin sulfone was considerably more effective as an antagonist for oxybiotin than for biotin. The most marked differential inhibition was observed at a level of 800 millimicrograms of biotin sulfone per tube. This level completely inhibited oxybiotin activity, while growth due to biotin was virtually unaffected (Fig. 1). Considerably higher sulfone levels had to be used in order to prevent growth due to biotin.

The inhibitory effect of biotin sulfone upon both biotin and oxybiotin was more pronounced at an incubation period of only 48 hours. With this shorter incubation, a marked differential inhibitory effect was noted at a level of 200 millimicrograms of biotin sulfone per tube.

A similar differential inhibition was observed with γ-(3,4-ureylenecyclohexyl)butyric acid. Levels of this compound ranging from 40 to 200 γ per tube were employed in the presence of *d*-biotin (0.1 to 1.0 millimicrogram per tube) or *dl*-oxybiotin (0.2 to 2.0 millimicrograms per tube). At incubation periods of 72 and 48 hours, the most marked differential inhibition was noted with 70 γ (Fig. 1) and 40 γ of the antagonist per tube, respectively. The inhibitory effect of the sulfone and the ureylene derivative upon *dl*-oxybiotin could be reversed by increasing amounts of the oxygen analogue.

It would seem possible to utilize the observed differences in sensitivity of biotin and oxybiotin toward biotin sulfone and γ-(3,4-ureylenecyclohexyl)butyric acid as the basis for a differential assay.

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THE OCCURRENCE OF A "PELLAGRAGENIC" AGENT
IN CORN

Sirs:

The time honored association of the occurrence of pellagra with the eating of corn is not, as Aykroyd and Swaminathan[1] have shown, due entirely to deficiency of nicotinic acid in this grain. Krehl *et al.*[2] have shown that corn exerts a growth-inhibiting action on rats receiving a ration low in tryptophane, and that nicotinic acid causes restoration of normal gains.

Response of Mice to Corn Fractions

Addition to basal ration		No. of animals	Average gain
	equivalent per cent of corn		*gm. per wk.*
1. None		37	2.1*
2. Nicotinamide, 0.2%		14	2.7
3. $CHCl_3$ + NaOH extract of corn	200	14	1.2
.	100	4	0.5†
	50	4	1.8
4. (3) + nicotinamide	200	11	3.0
5. Dilute H_2SO_4 extract of $CHCl_3$ extract	200	8	0.5
6. Acid residue of (3)	200	5	2.1

* The mice used in these experiments were a smaller breed than those used in previous work from this laboratory.

† Decrease in potency with more than 100 equivalent per cent of (3) was probably due to traces of nicotinamide. Removal of this by shaking (3) with water resulted in increased potency.

These authors have explained their observations on the basis of amino acid imbalances[3] and of the effect of corn on intestinal bacteria. On the other hand, as a result of experiments on the induction of nicotinic acid deficiency with 3-acetylpyridine,[4] Woolley has postulated that the pellagragenic action of corn may be due to a specific substance, possibly an analogue of nicotinic acid.[5] Attempts have therefore been made to demonstrate a substance in corn which would cause a pellagra-like disease preventable by nicotinic acid. The presence of such a pellagragenic substance

[1] Aykroyd, W. R., and Swaminathan, M., *Indian J. Med. Res.*, **27**, 667 (1940).

[2] Krehl, W. A., Tepley, L. J., and Elvehjem, C. A., *Science*, **101**, 283 (1945).

[3] Krehl, W. A., Sarma, P. S., Tepley, L. J., and Elvehjem, C. A., *J. Nutr.*, **31**, 85 (1946).

[4] Woolley, D. W., *J. Biol. Chem.*, **157**, 455 (1945).

[5] Woolley, D. W., *J. Biol. Chem.*, **162**, 179 (1946).

has been demonstrated and considerable concentration of it has been achieved. The basal diet for this purpose was composed of sucrose 85, casein 9, cystine 0.2, salts[6] 5, fortified corn oil[7] 1, and adequate water-soluble vitamins except nicotinic acid.[4] Although the addition of corn to this ration did not result in poor growth in mice as it did in rats, a chloroform and sodium hydroxide extract of the grain markedly reduced the growth rate. In addition, such an extract caused many of the animals to develop mild diarrhea, and considerable reddening of the skin and tongue. These signs were similar to those seen in pellagra and were preventable or curable with nicotinamide (0.2 per cent of the ration). Commercial crude corn oil in contrast to the alkaline chloroform extract was not deleterious.

The disease-producing agent appeared to be a weakly basic water-soluble compound, since it was extractable from chloroform by dilute H_2SO_4, and remained in aqueous solution when the sulfate was removed with barium hydroxide. Whether or not it is a pyridine derivative cannot be established until the pure material has been isolated. By appropriate solvent extractions an ether- and water-soluble fraction has been prepared which caused maximal inhibition of growth of mice under our conditions when fed at a level of 1 mg. per 100 gm. of ration (100,000-fold concentration of activity).

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[6] Phillips, P. H., and Hart, E. B., *J. Biol. Chem.*, **109**, 657 (1935).

[7] Woolley, D. W., *J. Biol. Chem.*, **143**, 679 (1942).

[8] With the technical assistance of A. Holloway and R. A. Brown.

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